A pilot study to establish and evaluate the CHUV assay as a cost-effective tool for human papillomavirus genotyping in HIV-infected women

Dissertation submitted as part of fulfilment for the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R.Medical University, to be held in April 2013

CERTIFICATE

This is to certify that the dissertation entitled, "A pilot study to establish and evaluate the CHUV assay as a cost-effective tool for genotyping human papillomavirus in HIV-infected women" is the bonafide work of Dr.Pallavi Ravindra Baliga toward the M.D (Branch –IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R Medical University, to be conducted in April 2013.

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Introduction

Cancer of the uterine cervix is one of the leading causes of cancer death among women worldwide. The estimated number of new cervical cancer cases per year is 500,000, of which 79% occur in developing countries. Cervical cancer is ranked highest or second-highest among cancers in women in developing countries. In India, every year 132,082 new cases of cervical cancer cases are diagnosed and 74,118 women die from the disease. Thus, India has one-fourth of the global burden of cervical cancer (WHO Global Statistics, 2010).

Studies in several parts of the world have demonstrated a very strong association between human papilloma virus (HPV) and cervical cancer. Persistent infection with human papilloma virus is now recognized to be a necessary although not a sufficient cause (1).

HPV is a small, non-enveloped DNA virus belonging to the family *Papillomaviridae*. Over a 100 different types of HPV have been identified from clinical specimens collected from humans throughout the world. Based on the type of epithelium infected, HPV is classified into 'cutaneous' and 'mucosal' types (2). Based on their association with ano-genital cancer, genital HPVs are further grouped into high-risk types (16,18,31,33,35,39,45,51,52,56,58,59,68,73,82), probably high risk types (26,53,66) and low-risk types (6,11,40,42,43,44,54,61,70,72,81 and CP6108). Worldwide and in India, HPV 16 and 18 are the most frequently detected types in cervical cancer (3).

Cervical cancer arises in the transformation zone of the uterine cervix. HPV infection is acquired predominantly at the beginning of sexual activity and shows peak prevalence in younger age groups (4). Most infections are of transient nature and clear spontaneously within 1–2 years and only rarely give rise to cancer (5,6). However, when infection persists, the viral oncoproteins produce perturbation of cell-cycle control resulting in cervical intraepithelial neoplasia (CIN), a precursor of cervical cancer.

Women who are immunocompromised may be at increased risk for persistent infection with human papillomavirus (HPV) resulting in an increased risk of cervical neoplasia (7). HIV-positive women also tend to have multiple HPV types and more non - HPV16/18 infections (8). HIV infection may increase risk for persistent HPV infection as well as promote reactivation of latent HPV infection (9). Since both HPV and HIV are sexually transmitted, these 2 infections commonly coexist (10).

Current cervical cancer screening strategies using cytology as the screening tool are effective but have its own limitations i.e. mainly poor sensitivity. HPV DNA testing is therefore a promising new technology for screening of women for cervical cancer. The greater sensitivity of HPV DNA testing compared to cytology argues strongly for using HPV DNA testing as the primary screening test in newly implemented programmes. The development of an affordable test for HPV DNA testing makes this a viable alternative to cytological screening (11).

The aim of this study is to evaluate the performance of a cost-effective reverse hybridisation assay (CHUV assay) in comparison to a commercial licensed reverse hybridisation assay (Linear Array, Roche).

AIM & OBJECTIVES

AIM: To establish and evaluate the CHUV assay as a cost-effective tool for HPV detection and genotyping in HIV-infected women.

OBJECTIVES:

1. To establish a cost-effective, in-house reverse hybridization Assay (CHUV assay) as a tool for HPV detection and genotyping in HIV-infected women.

2. To compare the accuracy indices of the CHUV assay with a licensed commercial assay (Linear Array, Roche Diagnostics).

Review of Literature

1. Cervical cancer:

1.1 Scenario in the world - Cancer of the cervix is one of the leading causes of cancer among women worldwide. The estimated number of new cervical cancer cases per year is 500,000. Of these around 80% occur in developing countries, due to lack of routine screening programmes. Mortality due to cervical cancer accounts for around 8% of all deaths worldwide (WHO HVP Statistics, 2010). The rates of incidence and mortality due to cervical cancer have declined over the last few decades in many western countries, primarily due to national screening strategies. In developing countries, the incidence and mortality rates have been relatively stable or have shown modest declines (12). The majority of cervical cancer cases are squamous cell carcinomas followed by adenocarcinomas.

1.2 Scenario in Asia and India - Cervical cancer comes second after breast cancer in the Asian continent with southern Asia having the highest number of cervical cancer cases.

In India, cervical cancer ranks highest among the cancers in women. It is also the most frequent cancer among all women and women between 15 and 44 years of age. Current estimates indicate that every year 1, 32, 082 women are diagnosed with cervical cancer and 74,118 die from the disease, the age-standardised mortality rate being 15% (WHO HPV Statistics 2010).

1.3 HPV and Cervical cancer: Cervical cancer is now known to be caused by the human papillomavirus (HPV). The initial associations of HPV with cervical cancer began in the 1970s where cases of condyloma acuminata were followed up and found to develop into cervical cancer (13). Definitive evidence was provided by large scale worldwide studies which showed that HPV is present in around 93% and subsequently in 99% of invasive cervical cancer (ICC) cases. Persistent infection with HPV is now recognized to be a necessary although not a sufficient cause for the development of cancer (1,14).

2. Human papilloma virus:

2.1 Classification and Taxonomy:

Historically, papillomaviruses (PV) were classified along with the Polyomaviruses under the Family *Papovaviridae*. Sequencing of the PV genome, however, indicated that, although PVs share a common genetic organization, they differ from polyomaviruses and have no major sequence homology to polyomaviruses. This led to PVs being grouped under a separate family, the *Papillomaviridae*, by the International Committee on the Taxonomy of Viruses (15).

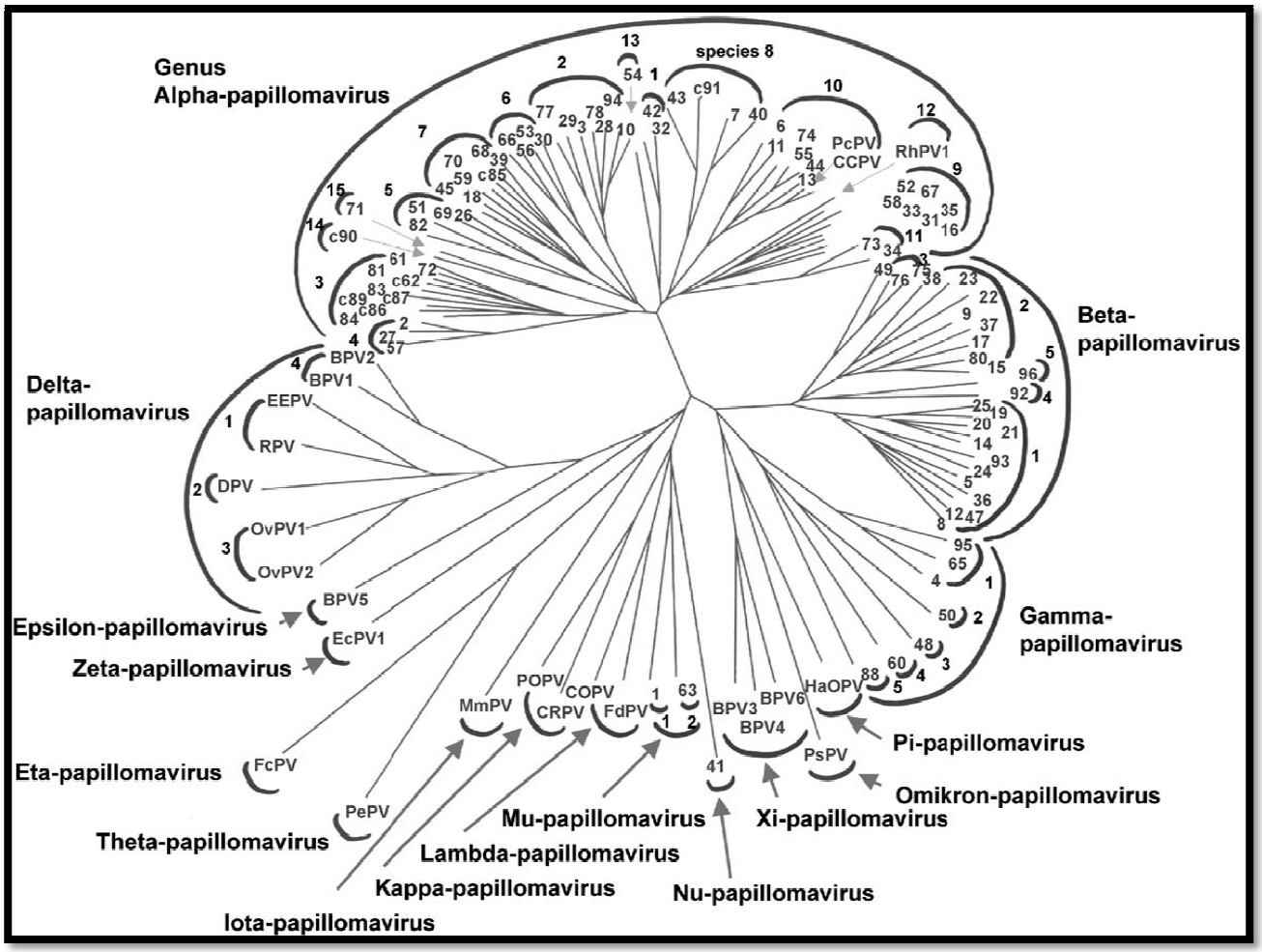
Papillomaviruses are currently classified based on differences in the L1 ORF into: genus, species, type, subtype, and variant. PVs are divided into 16 genera, each designated by a letter of the Greek alphabet (15). Within a given genus, the L1 DNA of all members share more than 60% identity and PVs showing 60% to 70% are classified as species. A viral type, within a species, has 71% to 89% identity with

other types. At present, 180 sequenced genotypes of HPV are known (16). Types are further classified into subtypes, which share 90% to 98% identity, and variants, which have more than 98% identity. More recently, variants have been described for a few types, most importantly for HPV 16 due to its wide prevalence and medical importance. Five phylogenetic clusters have been defined for HPV-16 based on sequence variation of the L1, L2, and LCR regions of HPV-16: European (E), Asian (As), Asian-American (AA), African-1 (Af1), and African-2 (Af2) (17).

The human papillomaviruses of medical importance belong to the genera alpha-Papillomavirus and beta – Papillomavirus.

Depending on the type of epithelium infected, HPVs are also classified as ‘mucosal’ and ‘cutaneous’ types. The members of Alpha genus primarily affect the genital epithelium and the non-genital mucosal epithelium while the beta-Papillomaviruses affect the non-genital skin (2).

HPV classification and taxonomy:



(Courtesy: de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H, Classification of papillomaviruses, Virology 324, p 17–27, 2004)

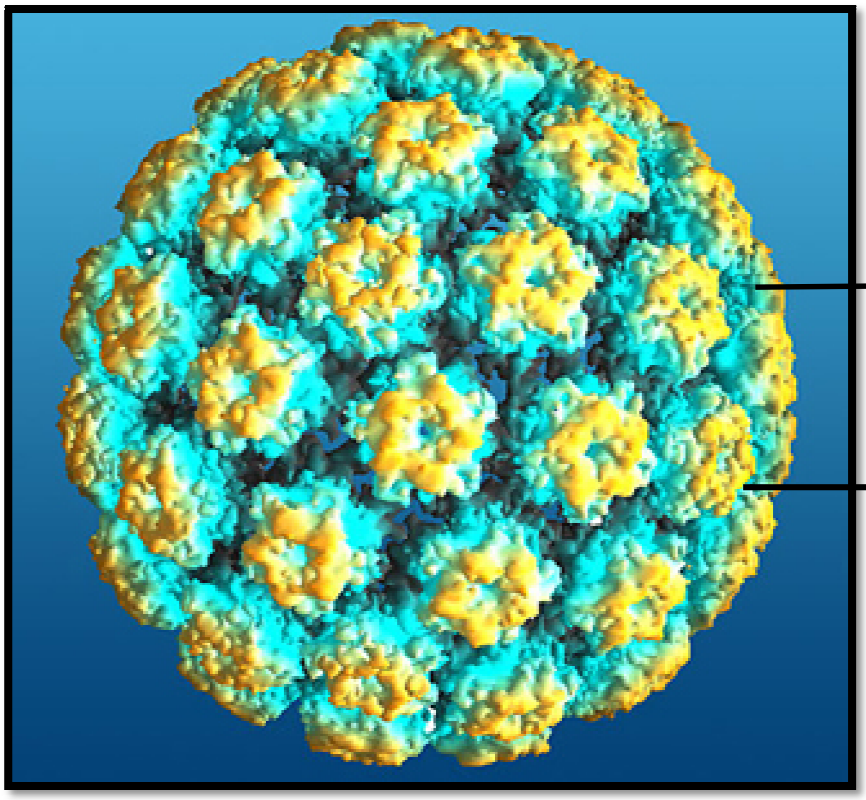
2.2 Structure of HPV:

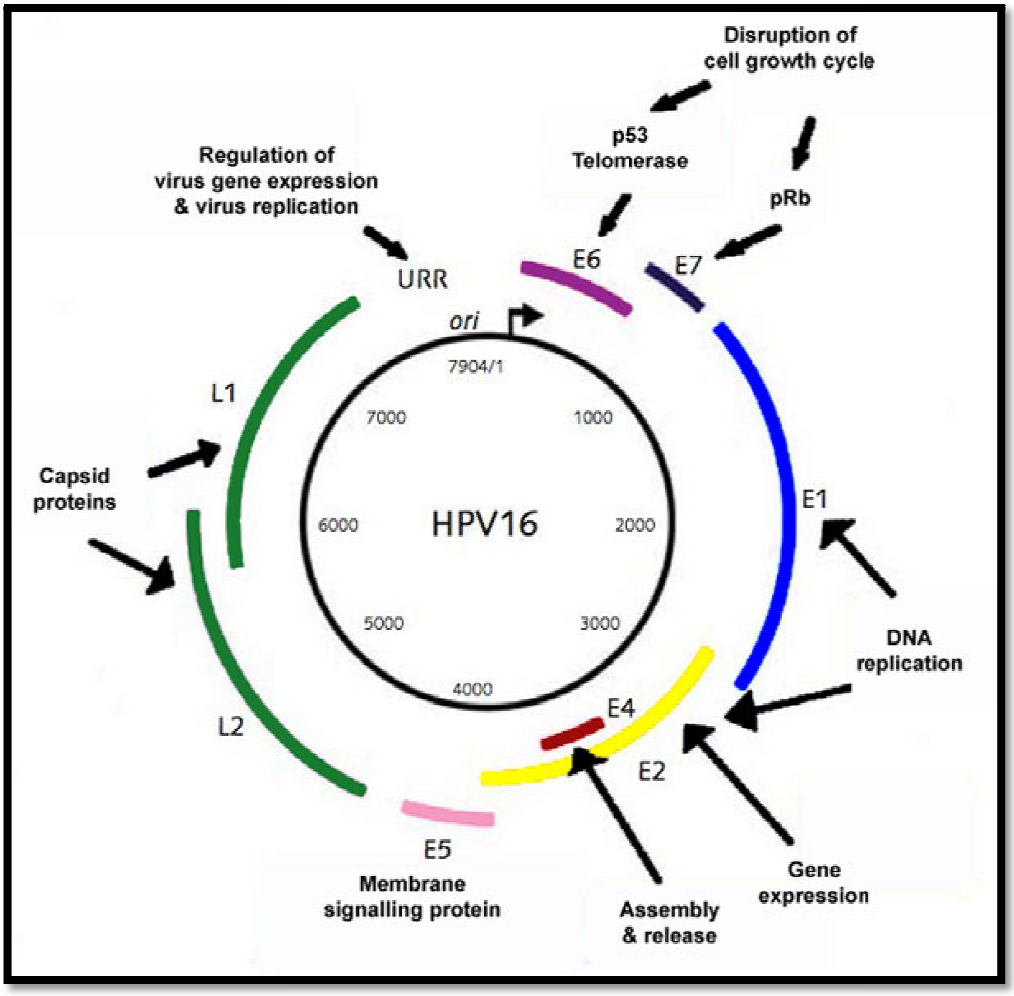
Papillomaviruses are small, non-enveloped, DNA viruses. The virion measures around 52 to 55nm in size and consists of a single molecule of double-stranded DNA, therefore belonging to Baltimore Class I. The genome is enclosed within an icosahedral capsid consisting of 72 capsomers (18). The capsid is made up of two

structural proteins – the major protein L1 and minor protein L2, both of which are virally encoded. The L1 protein constitutes 80% of the virion by weight (19,20).

The viral capsid encloses a double stranded DNA molecule made up of 8000 base pairs. The DNA in the virion has a supercoiled circular configuration. The putative open reading frames (ORFs) are located only on one DNA strand which acts the template for transcription. The coding strand can be functionally divided into two regions – the early region (E) and the late region (L) depending on their position in the genome. The early region codes for the viral regulatory proteins, including the viral proteins that are necessary for initiating viral DNA replication. The late ORFs lie downstream to early genes and code for the capsid proteins. L1 is the major capsid protein and the L2, the minor capsid protein is involved in DNA binding and encapsidation (21). The HPV genome also has a small region of about 1 kilobase pair size, between the early and late regions, that does not contain any ORFs and is known variously as the long control region (LCR), the upstream regulatory region (URR) or the non-coding region. This region contains the origin of DNA replication as well as important transcription binding sites. The three regions in the viral genome are separated by two polyadenylation sites –early pA and late pA (22).

Structure of the Human Papillomavirus:





2.3 Functions of the viral proteins:

2.3.1. E1 – E1 possesses DNA helicase activity (23).

2.3.2 E2 -This protein serves three major functions in the viral life cycle (23–25).

(i) It regulates the expression levels of the other viral gene products.

(ii) Initiates DNA viral replication with the help of E1.

(iii) During host cell division it plays a major role in the transfer of the viral genome to the daughter cells.

2.3.3. E4 – The exact function of this protein is still unknown, although it is expressed abundantly during viral replication. It is thought to aid viral DNA amplification and release (26). An E1-E4 complex is formed by the fusion of the first five amino acids of the E1 protein with E4; and is produced during the later stages of replication. The complex causes collapse of the cytokeratin network within the cell which helps in release of the virions from the infected cells (27,28).

2.3.4 E5 – E5, a membrane-associated protein, is important in the early course of infection. It regulates cell growth by forming complexes with growth-factor receptors. It has also been found to prevent apoptosis following DNA damage. However, in the process of carcinogenesis, during integration of the viral DNA into the host cell genome, the E5 gene gets deleted. So, E5 is not necessary in the late events of HPV-mediated carcinogenesis (29–31).

2.3.5 E6 – E6 along with E7 plays an important role in carcinogenesis. It interacts with and causes degradation of the tumor-suppressor protein p53. This results in resistance to apoptosis. It also prevents the degradation of the SRC-family kinases thus stimulating cell growth (32–34).

2.3.6 E7 – An important protein for HPV-mediated carcinogenesis, it targets cell-cycle regulatory pathways controlled by the tumour-suppressor protein Retinoblastoma protein, pRb. It thus provides an environment favourable to viral DNA replication by maintaining an *S*-phase like state in the differentiating keratinocytes (33,35,36).

E6 and E7 can individually immortalize cells but work more efficiently together (37,38).

2.3.7 Late viral proteins - L1 and L2 are the major and minor constituents, respectively, of the viral capsid. When overexpressed in eukaryotic cells, L1 can self-assemble to form virus-like particles (VLPs). These VLPs are the basis for prophylactic vaccines against HPV, through induction of neutralizing antibodies (26). L2 plays an important role in transport of the viral genome into the host cell nucleus (39).

2.4 Viral Replication:

The papillomaviruses are highly species-specific and have a specific tropism for squamous epithelial cells. The viral gene expression is linked to the differentiation state of the epithelial cell. The basal cell is the only differentiating cell in the squamous epithelium; therefore, it is the primary site of infection with HPV. However, maximum expression of viral proteins and viral shedding occurs in the upper differentiated layers of the squamous epithelium (40,41).

HPV infection is said to occur through microwounds in the basal layer of the squamous epithelium where it infects the keratinocyte stem cells (42). The receptor for binding is not clearly known although some of receptors implicated in pathogenesis include the heparin sulphate, cell surface glycoproteins and alpha-6 integrin receptor (43,44).

The virus enters the cell by clathrin-dependant receptor mediated endocytosis. Uncoating of the viral capsid with release of the viral genome occurs in the cytoplasm and with the help of the L2 protein the genome enters the nucleus of the host cell. Following infection, the viral DNA exists as a stable episome within the nucleus and amplifies to produce multiple copies. The virions are maintained at usually 20-100 copies/cell. Thus it establishes a non-productive state of infection (42). As the infected cells migrate upwards and undergo differentiation, cellular replication ceases. Differentiation – dependant late gene expression occurs along with capsid assembly and the production of numerous copies of viral DNA. This is known as the productive state of infection. Koilocytes, which are pathognomonic of HPV infection, are the sites of late gene expression, viral DNA amplification and capsid assembly (45,46).

Replication is totally dependent upon the cellular DNA synthetic machinery. The challenge for the virus is that the cellular DNA polymerases and other replication factors are produced only in mitotically active cells. To overcome this, HPVs encode the E6 and E7 proteins which inhibit the apoptosis and delay the differentiation program of the infected keratinocyte. This allows the viral DNA replication.

Transcription control –The transcription and translation of papillomavirus is controlled by two major promoters and many minor promoters.

Early gene expression – The first major promoters such as p97 in HPV16 and p105 in HPV 18, control the expression of the early genes and are expressed throughout the life cycle of the virus. These promoters consist of binding sites for various cellular factors essential for viral transcription. Early gene transcription is also regulated by the *cis*-transcriptional enhancer present in the URR in the genome that renders it epitheliotropic as it is activated only in differentiating epithelial cells. In high-risk HPV infection, a single promoter controls the expression of both the E6 and E7 transcripts, whereas in low-risk HPV infections, separate promoters are involved for E6 and E7 transcription (47).

The second major differentiation-dependant promoter is the late viral promoter, called p742 in HPV 31 and p670 in HPV 16 (48,49). It is seen in the E7 ORF and directs the expression of two sets of transcripts. The first transcripts encoded are the E1˜E4, E5 and E1/E2 proteins. The second set of transcripts that are encoded are the late viral genes, L1 and L2.

The cellular factors that control the late viral promoter transcription are currently unknown. However, induction of terminal differentiation and maintenance of the viral genome as an extrachromosomal element has been found to contribute to late viral promoter transcription (50).

Latency – HPV studies have shown a lag phase between infection of cells with HPV and the onset of viral replication. This 3-5 week lag before the viral gene expression probably reflects the time required for the infected stem cell to start dividing. It has been observed that viral DNA can be detected in the lower layers of infected cells only after 3-4 weeks of infection. This progressively moves upwards and by 5-8 weeks, numerous copies of viral DNA are detected in the upper superficial layers of the epithelium. Similarly, the E6 and E7 mRNAs are present at low levels in the lower layers but are abundant in the upper superficial layers (51). It is suggested that latency may result when inoculating titres are low (52,53).

2.5 Epidemiology of genital HPV infection:

2.5.1 Incidence & Prevalence – HPV is now an established cause of invasive cervical cancer throughout the world. Studies have shown that HPV is present in almost 100% of the invasive cervical cancer cases (1). In the general population, about 11.4% of women with a normal cervical cytology are estimated to harbour cervical HPV infection. The prevalence of cervical HPV infection varies from 14% in developing countries to 10 % in developed countries (WHO Global Statistics, 2010).

HPV prevalence is highest in women younger than 35 years of age, decreasing in women of older age. However, the age distribution of cervical HPV infection shows a

bimodal curve in most regions, with a first peak among young women (just after sexual debut), a lower prevalence plateau at middle age, and variable higher rates among older women (45 years) (54,55).

2.5.2 HPV genotypes - The most common HPV genotypes worldwide are 16 and 18 followed by the other high-risk types such as 31, 33, 35, 45, 52 and 58. Although the prevalence of HPV 16 and HPV 18 are similar in most regions, some geographic variation is seen with the non-HPV 16 types. HPV 31 is seen more commonly in Europe; HPV 52 is seen in North America, Africa and Asia and HPV 18 is seen in Indonesia (14). HPV 45 is less frequently seen when compared to the other types (54).

2.5.3 Association with cervical disease - The prevalence of HPV infection increases with the severity of cervical disease and HPV is present in 80-90% of high-grade lesions and 60-80% of low-grade lesions (56)(57). HPV 16, being the commonest type, accounts for 22% of HPV infection in HPV-positive women. HPV 16 and 18 contribute to 70% of invasive cervical cancers worldwide, the others types responsible for 20% of the cases (54). Worldwide, the rates of HPV 16 infection in ICC, HSIL and LSIL are 54%, 44% and 19% respectively. HPV 16 is more commonly (58% vs. 35%) associated with squamous cell carcinoma of the cervix while HPV 18 (12% vs. 38%) occurs commonly in adenocarcinoma (WHO Global Statistics, 2010).

2.5.4. HPV in Asia - A similar picture is seen in Asia – prevalence of HPV in the general population being 11% (WHO Asia Statistics,2010). HPV prevalence is noticed to remain constant across all age groups (58). HPV 16 and HPV 18 are the most common types accounting for 70% cases of ICC; followed by HPV 58, 33, 52, 45, 31,

35 and 39 in decreasing order of prevalence. HPV 16 occurs more commonly in SCC and HPV 18 in ADC (56,59).

2.5.5 HPV in India - In India, an overall prevalence of 12% is seen women with normal cytology (60). No significant variation is seen among HPV prevalence in North and South India. The most common types are HPV 16 and 18 followed by 45, 33, 35, 58, 59 and 31 (60). HPV types 16 and 45 are more common in North India, HPV 35 in South India and HPV 18 & 58 in East India (61). HPV is present in 94% of ICC cases, 79% of which are caused by the HPV 16/18 fraction. However, the HPV 16/18 fraction is responsible for 88% of ICC cases in North India when compared to 77% in the south. The type-specific prevalence of HPV-16, -45, -33 and -35 is higher in SCC than in ADC, whereas the type-specific prevalence of HPV-18 and -31 is higher in ADC than in SCC. HPV-18 is significantly more common in ADC (34.5%) than SCC (60). Single infection is seen in 70-80% and multiple infections in 14-18% of ICC cases (60). HPV variants are also important in pathogenesis of cervical cancer. The European variant of HPV16 is found to be most prevalent in India (85-90%) (62).

2.6 Transmission of HPV and risk factors for progression to cancer:

HPV is primarily transmitted by direct skin to skin contact or skin to mucosa contact. Epidemiologic studies clearly demonstrate the sexual mode of transmission in transmission of anogenital HPVs (63,64). Non-sexual routes of transmission have also been described and include fomites and vertical transmission (65).

Apart from sexual activity, other factors that can influence acquisition of HPV and progression to cervical cancer are:

2.6.1 Age and sexual activity – HPV infection is more common in young sexually active girls and is also related to the number of partners. Highest metaplastic activity at the squamocolumnar junction of the cervix occurs at a young age and may favor the acquisition of HPV (66,67). The risk of acquiring HPV increases with early age at first sexual intercourse, higher number of sexual partners, recent change in sexual partners, and a history of other sexually transmitted infections (68). The prevalence of cervical HPV infection decreases with age and is probably due to fewer partners and immunity to previously cleared infection.

2.6.2 Parity and use of contraceptives – Studies have shown that the higher parity and long-term use of oral contraceptives is associated with increased risk of development of cervical cancer (69,70). These appear to be significant co-factors in the development of cervical cancer. Use of condoms has been shown to reduce the risk of transmission (71).

2.6.3 Smoking or tobacco use – Smoking is an independent risk factor for progression to cervical cancer. Local immunosuppression induced by smoking and the carcinogens may be responsible for the same (72–74).

2.6.4 Immunosuppression – Studies have shown increased prevalence of HPV infection and cervical cancer in HIV-infected women. The poor cell-mediated immunity in these patients fails to clear the HPV leading to persistent infection

and progression to high-grade squamous intraepithelial lesions and cervical cancer (75,76).

2.6.5 Viral load and variants – The role of viral load in the progression of cervical cancer is unclear. The results of studies correlating viral load with cervical disease and its progression are variable. Studies have shown that higher loads of HPV 16 are associated with high-grade lesions (77,78). However, there are other studies that show that HPV viral load does not increase with advancing severity of disease (79). A study done by Gravitt et al. showed that high viral load of most high risk types is associated with prevalent infection, however, only high HPV 16 viral load is associated with incident infection (80). More recently, variants of HPV types have been discovered and their association with the severity of cervical cancer has been assessed. European variants of HPV 16 are found to cause a more aggressive form of disease compared to the Non - European variants which are associated with higher rates of persistent infection (81).

2.6.6 Other factors – Other sexually transmitted infections such as HSV-2, when present along with HPV infection can increase the chances of progression to cervical cancer (82).

There is some evidence of hereditary predisposition to the risk of cervical cancer. Certain HLA types are shown to protect against cervical cancer (83,84). In India, higher parity, increasing use of oral contraceptives and HIV co-infection may contribute to the burden of cervical cancer (WHO India HPV statistics, 2010).

2.7 Clinical manifestations of genital HPV infection: HPV infection can manifest in three ways:

2.7.1 Anogenital warts (Condyloma acuminatum) – These occur most commonly on or around the anogenital region in both men and women. Anogenital warts are typically associated with low-risk types HPV-6 and HPV-11. Other types like HPV-16 may also cause warts. Most are asymptomatic and have low carcinogenic potential (85). Lesions may spontaneously resolve, remain the same, or increase in size and number. Warts can be treated by ablation, excision, or topical agents such as 0.5% podophyllin or 5.0% imiquimod.

2.7.2 Latent/inactive infection – Patients are usually asymptomatic with no external lesions. Cytology is usually normal and HPV DNA may be detected in about 10% of the patients (41).

2.7.3 Active infection with hrHPV – Here, the virus causes changes in infected cells which result in cervical intraepithelial neoplasia and cancer or other HPV-related anogenital malignancies.

2.8 Natural history of genital HPV infection:

Genital HPV infection is said to be the most common sexually transmitted infection. Presently over 40 types of HPV are implicated in anogenital infections, only a subset regularly causing cancer. Based on their association with anogenital cancer, genital HPVs are further grouped into high-risk types (16,18,31,33,35,39,45,51,52,56,58,59,68,73,82), probably high risk types (26,53,66)

and low-risk types (6,11,40,42,43,44,54,61,70,72,81 and CP6108) (3). HPV 16 and HPV 18 are the most frequently detected high-risk types and are associated with high-grade lesions and cervical cancer. However, non-oncogenic types such as HPV 6 and 11 also contribute to the burden of HPV-related disease. They cause not only precursor cervical lesions and anogenital warts but also cutaneous lesions and respiratory papillomatosis (17,86). Genital warts are usually caused by HPV 6 and 11 and are highly contagious (87). The clinical consequences of HPV 6 and 11 infections also manifest more rapidly compared with high-risk HPV types as incident HPV 6 and 11 infections give rise to genital warts over a shorter time frame from first HPV infection compared to time taken for progression of hrHPV infection to high-grade CIN (88).

Genital HPV infection occurs with the onset of sexual activity. Peak prevalence of infection is seen in women 15 - 25 years of age and thereafter it declines, although in some populations it may remain constant or show a second peak in older age (55). The median age of onset of sexual activity, in India, is between 15 - 25 years, and the HPV prevalence is seen to remain constant over all age groups thereafter (WHO India HPV statistics, 2010). Most infections in young immunocompetant women clear after a transient period of 1-2 years (5,6). Persistence i.e. detection of HPV for a long duration is more uncommon than clearance of infection and occurs only in about 10-20 % of infected women. Munoz et al. recently proposed a new definition for persistence which is infection lasting more than the median duration which was around 9 years (89). Persistent cervical HPV infection is an important risk factor for the development of high-grade lesions and progression to cervical cancer. This is

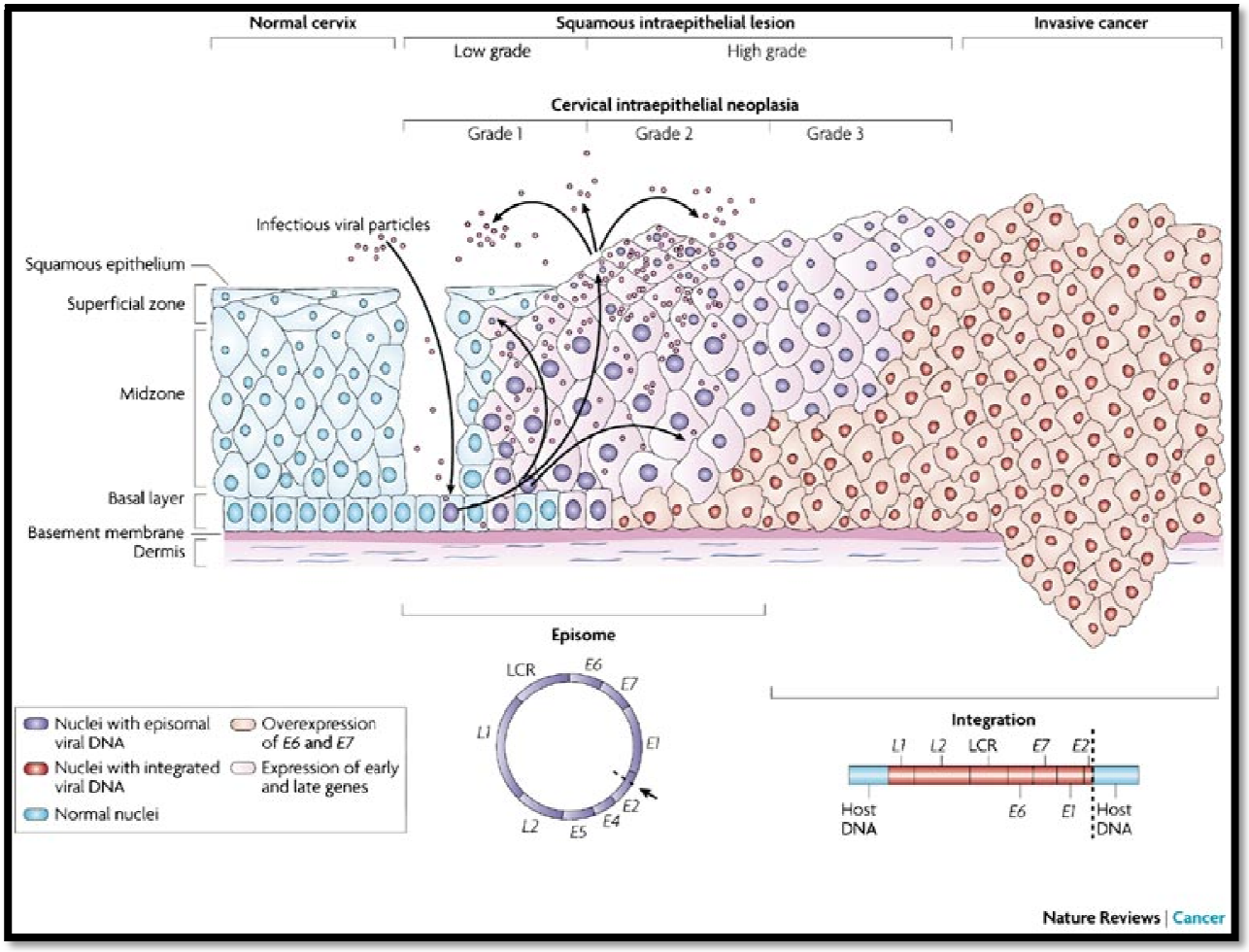
usually seen with the high risk types. Viral load may also influence persistence. Studies show that higher viral loads especially of the high risk types such as HPV-16 are associated with rapid progression to precursor lesions of cancer (89). However, persistence is not sufficient for carcinogenicity because there are non-carcinogenic types, like HPV 61, that persist without carcinogenic risk (90).

2.9 Natural history of cervical cancer-

Cervical cancer occurs most commonly in the transformation zone of the cervix. This region also known as the squamocolumnar junction is the junction between the columnar cells of the endocervix and the squamous cells of the ectocervix. Squamous cell cancer is more common type accounting for nearly 85% of cervical cancer followed by adenocarcinoma and a small number formed by neuroendocrine tumors (22). Precursor lesions typically undergo a series of dysplastic changes over many years during which time the squamous cells get replaced by basaloid cells (91). The extent to which the squamous epithelium gets replaced by basaloid cells determines the severity of the lesion, with the entire thickness being replaced in the most severe dysplasias. Histologically cervical dysplasias were classified as grades 1, 2 and 3 which correspond to mild dysplasia, moderate dysplasia, and severe dysplasia or carcinoma in situ. The newer Bethesda System classifies abnormalities as low-grade and high-grade squamous intraepithelial lesions (SIL) depending on the severity of dysplasia. Equivocal lesions are designated atypical squamous cytology of undetermined significance (ASCUS) for equivocal lesions (92).

The incidence rates of invasive cervical cancer tend to peak about 20–25 years after the peak age for HPV infection prevalence, and the incidence of cervical intraepithelial neoplasia (CIN) peaks in between. CIN may be caused by infection with either low-risk or high-risk HPV. Most dysplasias do not progress and, in fact, resolve spontaneously, with the chances of resolution decreasing with the severity of the dysplasia (93,94). Studies involving follow-up of women with precursor lesions, for many years, show that a section of women progresses to cancer if left untreated (95). The current view is that high-grade lesions can develop either from low-grade lesion or directly from a high-risk HPV infection (88,96–98). However, as described earlier, persistent infection with a high-risk HPV type, which occurs in a minority of infected women, is the single most important risk factor for developing CIN3 or invasive cancer. The rate of progression to cancer is found to be higher for HPV-16 than for HPV -18 (99).

Pathogenesis of cervical cancer:



Courtesy: Woodman C B J, Collins SI, Young LS. The natural history of cervical HPV infection: unresolved issues *Nature Reviews Cancer* **7**, 11-22 (January 2007).

2.10 Molecular basis of HPV-related carcinogenesis:

The human papillomavirus enters the dividing basal epithelial cells through microabrasions. Following infection, there is establishment of the viral genome as a stable episome (without integration into the host cell genome) in the cells. This requires the expression of the early viral proteins, E1 and E2. E2 plays an important role in productive infections as it – initiates viral DNA replication and genome

segregation and controls expression of the viral oncogenes E6 and E7. In the basal cells, therefore, a non-productive infection occurs with low copy numbers of the viral genome. As the virus moves up to the suprabasal layers, it switches to a rolling-circle mode of DNA replication resulting in production of high copy numbers of DNA (100).

The main event in the malignant transformation is the integration of the HPV DNA into the host genome. The ring form most often opens in the region of the E2 frame and a substantial part of the genome is deleted. This results in excess production of the E6 and E7 proteins. The ability of malignant transformation is seen with the E6 and E7 proteins of only the high-risk types (101,102). The events that follow leading to carcinogenesis are:

- E6 interacts with and causes degradation of the p53 protein which results in resistance to apoptosis. It also stabilises the SRC-family of kinases resulting in stimulation of cell growth. INK4A, a cyclin-dependant kinase counteracts these functions of E6 (32–34).

- E7, on the other hand, interacts with and degrades the pRB protein and causes genomic instability. The release of E2F complex from degraded pRB causes cell cycle progression. However, it may also hasten the apoptosis of the cells.

- E6 and E7, therefore, work synergistically to cause cell immortalization - E6 prevents apoptosis that is induced by high E2F levels, and E7 rescues E6 from inhibition by INK4A. (103).

Eventually, mutations accumulate that lead to fully transformed cancerous cells. Progression to cancer, seen with high-risk genotypes like HPV 16 and 18, generally takes place over a period of 10 to 20 years.

3. HPV and HIV:

Immunity is one of the major determinants in the natural history of cervical neoplasia. Studies from around the world have consistently documented a higher prevalence of cervical HPV infection in women who are HIV-positive (104). Women who are immunocompromised are at an increased risk for persistent HPV infection and cervical cancer. Immunosuppression caused by HIV-infection is also associated with a high prevalence of CIN and a high rate of persistence and progression of these lesions (9,10).

The burden of both cervical cancer and HIV-infection are highest in the developing countries. India is estimated to have around 2.5 million people infected with the HIV as well as a high incidence of ICC (105). Studies from India have shown a higher prevalence of HPV in HIV-infected women (39%) compared to in HIV-negative women (13%) (8). HIV-infection may cause persistence of HPV infection or reactivation of a latent infection. Morover, both infections are sexually transmitted and have similar risk factors for acquisition of infection (10,106). High prevalence and persistence of oncogenic HPV genotypes, infection with multiple HPV types, greater diversity of types and infection with non-16/18 types are common features in HIV-seropositive women (75,107,108). Women with HIV-infection are living longer due

to Anti-Retroviral Therapy (ART); yet, access to screening for prevention of invasive cervical cancer (ICC) remains inadequate. Thus, HIV-infected women remain at increased risk for HPV infection and cervical precancerous lesions progressing to ICC (109).

4. Non-genital manifestations of HPV infection:

4.1 Skin warts: These are benign papillomas that occur most commonly on the hands and feet, although they can arise in almost any location and are usually associated with low-risk HPV types such as HPV-1, 2, 3, 5, 8, 10, 27, 57 and 65. Lesions mainly cause cosmetic nuisance, however, most lesions regress spontaneously and regression is thought to be immunologically-mediated (110).

4.2 Epidermodysplasia verruciformis (EV): Epidermodysplasia verruciformis is a rare disorder in which affected individuals have a unique susceptibility to cutaneous HPV infection. The warts usually develop in childhood, become widespread, do not tend to regress, and, in some instances, may progress to squamous cell cancers. The HPV types associated are HPV-3, HPV-5, HPV-8 and HPV-10. One-third of these patients may progress to cancer, the most oncogenic types being HPV-5 and HPV-8 (22,111).

4.3 Non-melanoma skin cancer (NMSC): The squamous cell carcinoma (SCC) variant of NMSC is associated with HPV. The consistent finding of HPV in SCC associated with EV makes it a potential etiologic agent in these cases. The HPV types commonly found are those associated with EV (112).

4.4 Respiratory papillomatosis: This condition, mostly occurring in children, is characterised by papillomas in the larynx. Transmission likely occurs at the time of birth. Rarely, adults may be affected and acquire it through close sexual contact. The lesions may compromise the airway and have to be removed surgically. HPV-6 and -11 are implicated in this condition (113,114).

5. Association with other cancers:

5.1 Genital cancers: Genital HPV can routinely infect other genital areas that contain stratified squamous epithelium such as vulva, vagina and penis. The risk factors and HPV types associated with these cancers is similar to that of cervical cancer. Vulvar and vaginal dysplasia is seen to occur more frequently in women with a previous history of cervical dysplasia (115).

5.2 Anal cancer: Anal cancer is increasingly being reported in men worldwide. It is sexually transmitted and is more common in homosexual men and in HIV-infected persons. The rate of anal infection by HPV is similar to HPV cervical infection. As with cervical cancer, high-risk HPV have been found in most anal cancers, with an even greater preponderance of HPV-16 than in cervical cancer, and most anal cancers arise in the transition zone between columnar and squamous epithelium (116,117). Precancerous changes precede the development of anal carcinoma raising the possibility that like cervical cancer, anal cancer can be prevented. Prevention efforts, including HPV vaccination, could therefore be targeted to high-risk groups (118).

5.3 Head and neck cancers: Studies have consistently shown the link between HPV and certain subsets of head and neck cancer. Most of these HPV-associated cancers are located in the oropharynx, which includes the tonsils, tonsillar fossa, base of the tongue, and soft palate. HPV-16 accounts for about 90% of these cancers (119–121).

6. Diagnosis of HPV infections:

Studies have clearly shown the etiologic role of HPV in cervical cancer. Infection with high-risk HPV types results in precursor lesions which can progress over many years to develop into cancer. Conventional cytology can be used to detect the malignant changes in cervical cells but it is not very sensitive. HPV DNA detection therefore plays a key role in diagnosis. HPV cannot be efficiently grown in culture and HPV DNA detection by molecular methods forms the mainstay of diagnosis.

6.1 Conventional methods – Conventional methods are indirect methods as they only detect the sequelae of HPV infection; but are widely used for screening.

6.1.1 Pap smear - The primary method for diagnosis is still the Pap smear. The method was introduced by pathologist George Papanicolou, in 1949, before the cause of cervical cancer was known (122). Pap smear currently looks for changes in the cells of the transformation zone of the cervix, caused by infection with HPV. HPV infected cells known as ‘koilocytes’ usually show vacuolation with a pyknotic nucleus and a perinuclear halo. The reporting of Pap smear has changed over the last many years. Currently, the Bethesda system is followed which classifies the cervical changes into four categories –

i) ASCUS - Atypical Cells of Undetermined Significance ii) LSIL- Low grade squamous intraepithelial lesion iii) HSIL – High grade intraepithelial lesion iv) Invasive cancer (92). Cytology is inexpensive, easy to perform, has good specificity and is used widely for screening of cervical cancer. However, inadequate and improper sampling, high rates of false-negative results and technical errors are the limitations of the Pap smear.

6.1.2 Monolayer cytology/ Liquid cytology - To overcome this, monolayer cytology was introduced. Here, instead of directly smearing the sample onto glass slides, the sample is preserved in a solution after collection with a cytobrush. The morphology of cells is maintained, sampling is adequate and uniform and fewer false-negative results are seen. Liquid-based cytology has also significantly improved the rates of detection of dysplasias (123–125). Presently, FDA-approved automated systems are also available for interpretation and reporting of smears.

6.1.3 Visual inspection with 3% acetic acid or Lugol's Iodine - This method employs Lugol's Iodine or 3% Acetic acid for visualisation of changes in the cervix. Dysplastic or precursor lesions in the cervix will appear light yellow (saffron yellow/mustard yellow) or white respectively. Low cost, simplicity of the technique and good sensitivity are the advantages; while subjective interpretation and poor specificity limit the use of this test (126).

6.1.4 Colposcopy and biopsy – Patients with an abnormal cytological test are subjected to colposcopy and colposcopy-directed cone biopsies, which is considered the gold standard for cancer and HPV detection. Colposcopy is useful in detecting dysplasias and biopsies confirm diagnosis as characteristic pathologic changes are seen in the tissue. In addition, immunohistochemical stains can be used to detect HPV DNA or antigens in the tissue (17).

6.2 Immunohistochemical staining for p16INK4a – Infection with high-risk HPV types results in overexpression of p16INK4a. This may therefore be a good marker for infection by these HPV types. Monoclonal antibodies to detect p16INK4a in cervical tissues have been developed which allows precise identification of even small CIN or cervical cancer lesions in biopsy sections (103).

6.3 HPV DNA detection – HPV DNA detection is used as an adjunct to conventional cytology. Definitive diagnosis of cervical dysplasias and cancer are now based on detection of HPV DNA in the samples.

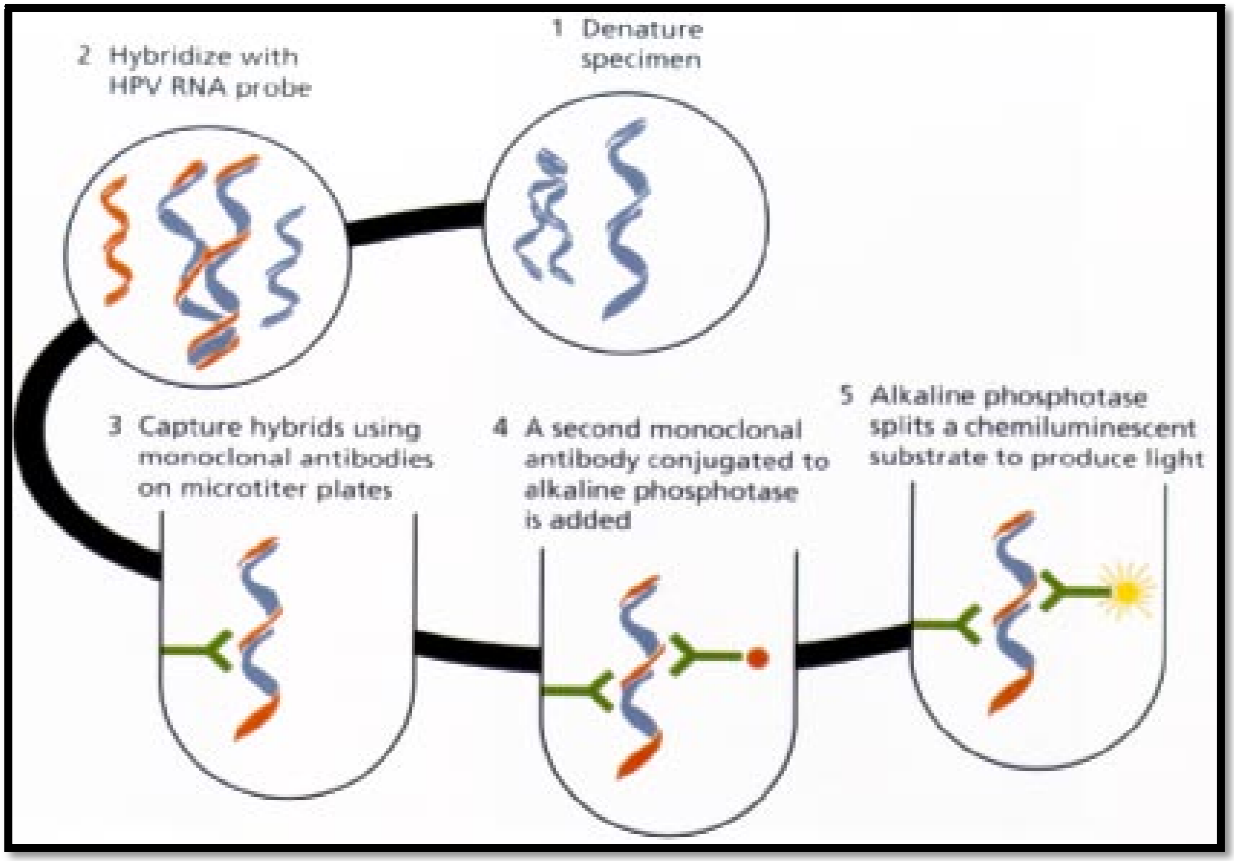
The assays available for HPV DNA detection are signal amplification, target amplification of conserved regions, and in situ hybridisation.

6.3.1 Signal amplification assays:

6.3.1.1 Hybrid Capture (HC2) assay - This is an FDA-approved assay and is widely used for detection of HPV DNA in exfoliated cervical cells. Cervical samples are collected using a cytobrush in a preservative solution. The HPV DNA present in the biological specimen is hybridized with two separate RNA probe mixes – one containing probes against 13 high-risk oncogenic (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and the other against 5 low risk non-oncogenic (6, 11, 42, 43, 44) HPV types. The resulting HPV DNA-RNA hybrids are captured and immobilised onto wells of a microtiter plate. Alkaline phosphatase- labelled anti DNA-RNA hybrid antibodies is added and the plate is washed. Addition of a chemiluminescent substrate results in a glow reaction if the sample is positive. The glow is measured using a luminometer and analyzed by a computer to give results in terms of Relative Light Units (RLU). RLU reflects the amount of DNA present in the sample and hence gives a semi-quantitative measure of the viral load. The HC2 assay cannot detect the exact genotype in the sample, however the result indicates if the sample contains high-risk or low-risk HPV. Recently, Hybrid Capture HR test which detects only the high-risk HPV – 16, 18 and 45 has been developed, which is useful as a screening assay (127). The HC2 assay is easy to perform in clinical settings, is reproducible and suitable for automation. The high-risk mix alone may be used to reduce the cost and time of the test. The main limitation of the HC2 assay is the problem of cross-reactivity of the high-risk mix with certain low-risk types which results in false-positive results and reduces the specificity of the test (128,129). It also cannot discriminate multiple

infections or detect novel HPV types; and there is no control for cell adequacy in the specimen.

Principle of Hybrid Capture2 assay:



6.3.1.2 Other signal amplification assays - Recently, two more FDA-approved assays based on signal amplification are available. The Cervista HPV HR test and the Cervista HPV 16/18 assays are based on Invader chemistry and are carried out in two simultaneous isothermal reactions – the primary reaction involves the binding of specific oligonucleotides to target DNA sequence of HPV; and the secondary reaction where a fluorescent signal is emitted which gives a measure of the HPV in the sample. Cross-reactivity with low-risk types is a drawback of this method.

6.3.2 Target Amplification methods –

PCR has been used for many years for the diagnosis of HPV in cervical specimens and is now considered the gold standard for HPV detection. The principle involves amplification of a conserved segment of the HPV genome using specific primers. Detection of amplified products is done by gel electrophoresis, enzyme immunoassay or line blot hybridisation.

6.3.2.1 PCR systems with consensus primers: The consensus primers target a conserved region of the HPV genome that is common to all the different types. Since the L1 region is most conserved, primers targeting this region are used.

6.3.2.1.1 MY09/11 and PGMY09/11 primers – The degenerate MY09/11 primers and modified PGMY09/11 primers target a 450bp region of the L1 gene. The PGMY primers were designed to improve the sensitivity of detection of HPV DNA and comprise two primer pools – an upstream pool with 5 oligonucleotides (PGMY11) and a downstream pool with 13 oligonucleotides (PGMY09). Comparison of the MY09/11 and the PGMY09/11 primers has shown an overall agreement of 91.5%. However, the PGMY system is found to be more sensitive, can detect an additional HPV types and multiple infection in clinical samples (130). The assay is, however, intended to detect a broad spectrum of genital HPV genotypes, so the specificity depends on the genotypes included in the analysis. When combined with the reverse line blot assay to detect around 39 genotypes, this primer system is an efficient tool for genotyping HPV and detection of multiple infection.

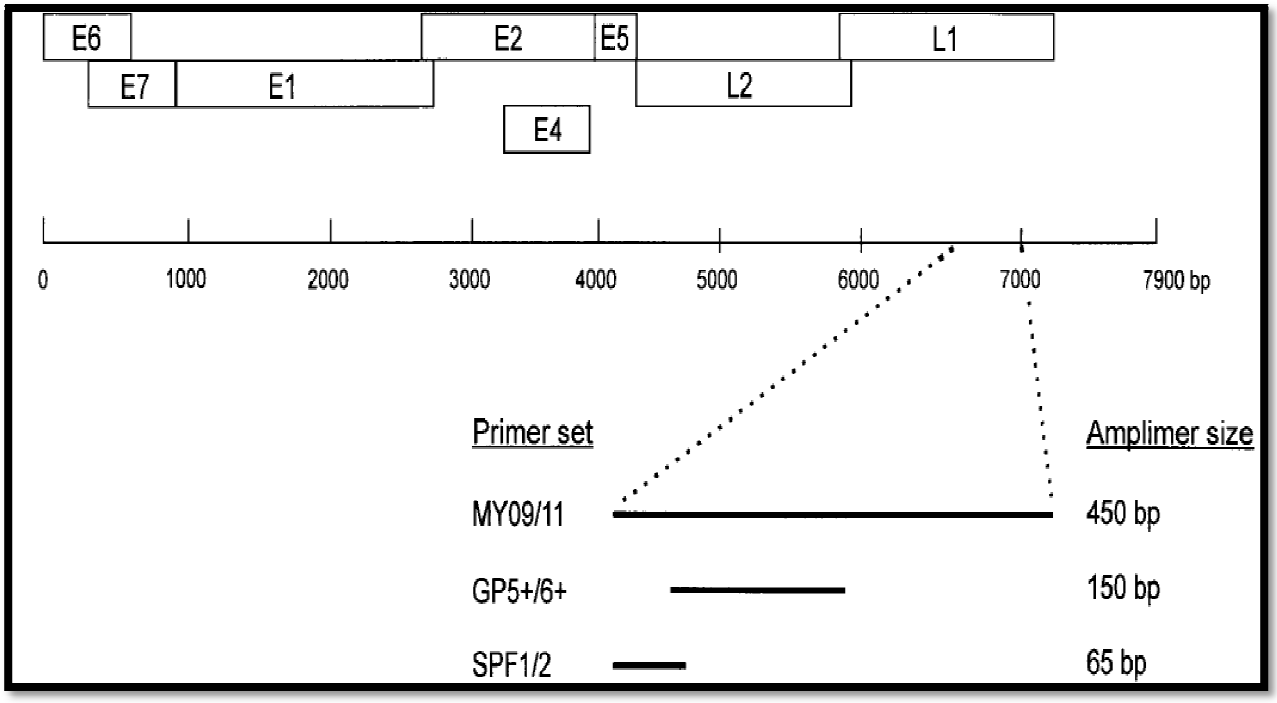
6.3.2.1.2 GP5+/6+ PCR system was developed as a refinement of the original GP5/6 system, and amplifies a 150bp region of the L1 region. It can amplify 14 high risk HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and 6 low risk types (HPV 6, 11, 40, 42, 43, and 44) and the amplified products are detected by microplate enzyme immunoassay (EIA). These primers are found to be 10 -100 times more sensitive than the original GP5/6 primers, having an analytical sensitivity at the femtogram (fg) level for highly complementary HPV types (131). Newer broad-spectrum primers BSGP5+/6+ have been designed to cover the HPV types not detected by the GP5+/6+ primers (132).

6.3.2.1.3 SPF10 PCR - This short fragment PCR system targets only a small 65 bp conserved region within L1 gene. The SPF system allows for detection of 43 HPV types and the amplified products are detected using a reverse hybridisation line probe assay (LiPA). In addition to detection of HPV in cervical scrapings it can be used for cervical tissue specimens as well (133). Although it is expected that this primer set would have enhanced sensitivity because PCR efficiency is inversely related to the size of the region amplified, the results between various investigations comparing primer sets are conflicting. Whereas some studies show an improved sensitivity of the SPF primers, others do not support a higher sensitivity of the SPF-InnoLiPA system (134,135).

The GP5+/6+ and SPF10 primers can be used can be used for HPV detection in paraffin embedded tissue as well.

6.3.2.2 Type specific PCR - More recently, primers targeting the E6 and E7 regions of specific HPV types have been constructed. These are highly specific as they are based on sequence variations in E6 and E7 genes and can help diagnose advanced disease as the E6/E7 oncogenic regions are integral in the evolution of cancer (136). Studies evaluating the use of E7 primer pool have found it useful to detect a spectrum of high-risk HPV types (137). The only advantage of these primers is that specific HPV types are identified without the need for separate genotyping. However, they are not practical for use in epidemiological studies due to the multiplicity of the genotypes present in clinical samples.

Schematic representation of different primer sets for HPV DNA detection. Primers targeting the L1 gene of HPV are the most widely used. ***(Courtesy: Kleter B, J Clin Microbiol. 1999 Aug; 37(8):2508-17)***



6.4 Post-amplification assays for HPV detection and genotyping:

HPV genotyping is essential for stratifying patients with risk of progression to cancer. Following amplification of HPV DNA, genotyping methods that can be employed are nucleotide sequencing, restriction fragment length polymorphism and nucleic acid hybridization.

6.4.1 Direct sequencing - Sequencing can be performed on PCR products and the sequencing data used to identify specific genotypes by a BLAST search for homology to existing sequences in the database (138). This is a labour-intensive method, expensive and is not routinely performed for HPV genotyping in clinical samples. Another disadvantage of direct sequencing is the lack of sensitivity in detecting multiple HPV types in a single sample (139). This technique may be employed in yielding sequence information on as yet uncharacterized HPV genotypes.

6.4.2 Restriction Fragment Length Polymorphism (RFLP) – RFLP requires the digestion of PCR products with various restriction endonucleases that cut DNA at specified base pairings. Each endonuclease digest results in fragments of differing sizes that confer a unique banding pattern for specific HPV types that can be detected by gel electrophoresis. RFLP is inexpensive, however, has to be done manually and is labour intensive. Because RFLP is a manual technique, variations can occur between each experiment (140). RFLP data may be difficult to interpret especially in infection with multiple types.

6.4.3 Nucleic acid hybridisation assays – Nucleic acid hybridization is probably the most commonly used method for HPV typing. Hybridisation can be performed with oligonucleotides immobilized at defined positions on various solid supports, such as strips and filters, microsphere beads and microarrays.

6.4.4 Line probe assays - Line Blot Assays (LBAs), based on amplification of conserved regions of HPV followed by hybridization to type-specific probes on line blot strips, are widely used. Hybridization is dependent upon a colorimetric reaction and hybridization at a specific line on the strip defines the HPV type. The number of individual HPV genotypes that can be determined varies with the specific manufacturer of the strips. Two commonly used line probe assays are – the Roche Linear Array genotyping which employs PGMY09/11 primers and the InnoLiPA line probe assay which uses SPF10 primers for DNA amplification. Most studies comparing these assays conclude that there is a high degree of correlation between methods for observing single infections. Some have found the INNO-LiPA test (Innogenetics, Temse, Belgium) has a greater sensitivity for multiple infections due to the short PCR products generated by the SPF10 primers used in the assay. However, most studies find the Linear Array better at detecting multiple infections. The LA test is still relatively expensive and may prove cost-effective only in high-throughput laboratories (133,139,141–143).

Many such assays are available which are based on PCR followed by probe hybridisation. Some of the commercially available assays include - Papillocheck from

Greiner Bio-One, CLART from Genomica, DNA chip from Biocare, PCR Luminex from Multimetrix and HPV genotyping LQ from Qiagen. Due to the need for expensive instruments required by these assays, they are unsuitable for use in a low-resource setting..

6.4.5 DNA Microarray systems –

Microarray systems work on the principle of hybridisation between immobilized HPV type-specific oligonucleotide probes on a glass slide with labelled PCR products. The results are read using colorimetric or fluorescence detection systems. In the case of multiple infections, multiple hybridization signals can be seen (144). Many microarray systems are available but the clinical utility of these systems has not been evaluated. Nevertheless, these methods have the potential to concurrently analyze and test for all HPV genotypes, sequences and mutations within the context of genetic factors such HLA haplotype or mutations within tumor suppressor genes (145).

6.4.6 ELISA/EIA – Another HPV detection system is the Enzyme Immunoassay where following PCR amplification with biotin-labelled consensus primers, HPV amplicons are captured on streptavidin-coated microwell plates and detected with a digoxigenin-labelled HPV generic probe mix. Roche Amplicor HPV test which has been widely used, is based on this principle, and can detect 13 high-risk HPV types.

6.5 HPV Quantification:

6.5.1 Viral Load – Viral load quantitation is done by Real-Time PCR systems which rely on the detection and quantitation of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. The FDA-approved Cobas 4800 test specifically identifies HPV types 16 and 18 while concurrently detecting the 12 remaining high-risk types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) at clinically relevant infection levels (146).

6.5.2 HPV mRNA detection – Detection of E6/E7 mRNA transcripts in the cervical samples is a reflection of the actual ongoing carcinogenesis. The recent FDA-approved Aptima HPV assay is a transcription-mediated amplification based test, which allows the detection of E6/E7 mRNA transcripts of 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68). This fully automated assay, however, does not discriminate between the 14 high-risk HPV types. No cross-reactivity between the HPV types is seen and has high sensitivity and specificity (147).

6.6 Serology – Serology can be used as an indirect determinant of past or present infection and progression to cancer. Recombinant technologies have allowed the generation of Virus like Particles (VLPs) that display conformational, type-specific epitopes of purified, correctly folded early proteins; and of infectious pseudovirions that are suitable for neutralization assays. Antibodies to capsid antigens can be tested

using various forms of ELISA where VLP acts as the antigen – eg. Direct ELISA, capture ELISA (148). Serological assays have been assessed in comparison with the detection of HPV DNA in the cervical tract. Most studies report the presence of antibody response in atleast 50% for women who were HPV 16 DNA positive (149). Neutralization assays are also available and are based on the ability of antibodies in patient's sera to prevent infection of cells by neutralising pseudovirions. These assays are said to be more type-specific and help quantify protective antibodies (150).

7. Clinical utility of HPV DNA testing-

Current screening strategies with cytology have been used for decades; yet, cervical cancer remains the leading cause of cancer in women in developing countries. Cytology as a screening tool is effective but has its own limitations i.e. poor sensitivity. The greater sensitivity of HPV DNA testing compared to cytology argues strongly for using HPV DNA testing as the primary screening test in newly implemented programmes (11).

Persistent infection with HPV is now known to be a necessary cause of cervical cancer. As HPV cannot be cultured and serology is unreliable as a marker of disease, HPV DNA detection remains the mainstay of diagnosis. Studies comparing HPV DNA detection with cytology have found significantly higher rates of detection of precursor lesions in women (151).

HPV DNA testing can be used in four scenarios:

7.1 Primary screening test – Studies have clearly shown the benefit of HPV DNA testing as a primary screening tool for cervical cancer, where a significant reduction in the lifetime risk of developing disease was seen. A large-scale study by Sankaranarayanan et al. clearly showed that, in a low-resource setting, a single round of HPV testing was associated with a considerable reduction in the numbers of advanced cervical cancers and deaths from cervical cancer (152) .

7.2 Adjunct to cytology for screening – When used alongwith cytology as screening tools, the rates of detection of precursor lesions and cancer significantly improved. Also, negative results with both the tests, reassures the women and repeat testing is avoided.

7.3 Triage of women with equivocal lesions (ASCUS) or LSIL – Patients with ASCUS or precursor lesions are usually referred to colposcopy to confirm disease. HPV DNA testing in patients with ASCUS would reduce the number of referrals to colposcopy if found to be negative. However, studies show that in patients with LSIL, HPV testing is not of value as it cannot distinguish between clinically significant lesions from non-significant ones (153,154).

7.4 Follow-up post-treatment – Here, the utility of HPV DNA testing would be to predict the chances of persistence or recurrence of disease in patients who have undergone ablative or excisional techniques for treatment of cervical cancer.

The development of an affordable HPV DNA test makes this a viable alternative to cytological screening. In India, use of HPV DNA testing as a primary screening test or as an adjunct to cytology would significantly reduce the frequency and interval of testing.

8. HPV Vaccines –

HPV vaccines are of two types - Prophylactic vaccines and Therapeutic vaccines.

8.1 Prophylactic vaccines – To date, two prophylactic vaccines have been developed and tested in large multicentric trials. Both are based on the recombinant expression and self-assembly of the viral protein L1 into VLPs. The HPV VLPs contain no DNA and hence are noninfectious. Since the vaccines are injected intramuscularly, there is rapid access of the VLPs to blood vessels and lymph nodes; the VLP's conformational epitopes induce neutralising antibodies. The injected HPV VLPs thus elicit a strong and sustained type-specific response. The quadrivalent vaccine Gardasil (Merck & Co.), protects against HPV 6, 11, 16 and 18 and the bivalent vaccine Cervarix (GlaxoSmithKline), protects against HPV 16 and 18. Both the vaccines are found to significantly reduce the incidence of cervical disease (155–158). Cross protection against other high-risk types is also seen, however, the neutralising antibody titres are much lower for these types (159). These two vaccines have been licensed for use in girls between 9-26 years of age and confer protection against genital warts, cervical cancer and its precursors, and other HPV-related conditions. Most studies demonstrate a protective efficacy of 95% and high immunogenicity with 100% seroconversion (155,160).

8.2 Therapeutic vaccines – The long gap between HPV infection and development of cervical cancer allows for intervention with therapeutic vaccines at many stages. E6 and E7 proteins are potential targets since they are expressed throughout the lifecycle of the virus (161). Vaccines containing E6/E7 peptides and DNA vaccines with the oncogenes have been evaluated and found to elicit a robust cell-mediated immune response (162,163). Chimeric vaccines are VLPs containing the capsid proteins L1/L2 fused with the early protein peptides. These induce both humoral as well as cell-mediated immune responses (164).

MATERIALS AND METHODS

Study design: This is a prospective study of diagnostic accuracy conducted in the Department of Clinical Virology, Christian Medical College and Hospital, Vellore. The institution is a tertiary care centre comprising a 2400 bedded hospital with an outpatient turnover of around 5000 cases per day. The clinical virology laboratory receives more than 3, 00, 000 samples every year. Molecular methods are performed for routine diagnosis of viral infections and research purposes. Presently, diagnostic testing for HPV (done on request) includes HPV DNA detection by PCR and genotyping, if positive, by sequencing.

Study duration: This study was conducted over a period of 17 months, from March 2011 to July 2012.

Study samples:

A. Clinical samples:

Consecutive 50 HIV-positive women attending the Infectious Diseases clinic and Obstetrics and Gynaecology outpatient departments in CMC, Vellore were recruited based on the following inclusion and exclusion criteria. Cervical samples were collected from the patients after prior consent.

Recruitment of study subjects:

Inclusion criteria for recruitment	**Exclusion Criteria for recruitment**
HIV seropositive women (Preferably newly diagnosed)	HIV seronegative women
Women between 18-45 years of age	Pregnant women
Sexually active women	-

The study group included women who tested positive for HIV antibody by atleast two 'rapid' HIV antibody detection tests. A pelvic examination was done, as is the management protocol for newly identified HIV-positive women. The samples for Pap smear and a cervical brush sample were collected in the Department of Obstetrics and Gynaecology, CMC, Vellore. Pap smears, done free of cost for the patient, were collected in ThinPrep Pap Smear liquid cytology bottles (Cytyc Corp., Boxborough, Massachusetts) and sent to the department of Pathology for reporting. The purpose of the cervical brush sample was explained to the patients, informed consent was taken and clinical details were filled in a proforma. CD4 counts were also done free of cost for the patients which was collected within two weeks of collecting the cervical brush sample. Samples for CD4 counts (3ml) were collected between 8 – 10 AM in the Department of Clinical Virology.

HPV DNA was collected using the DNAPAP cervical sampler (Qiagen, Gaithersburg, USA) which consists of a cytobrush (Christmas tree brush) in an STM medium. The

cervical brush samples were transported in an ice bucket kept at 4^{o}C, aliquoted into 1.5 ml appendorfs and stored at -20^{o}C until further use.

B. WHO Reference samples:

Forty-three samples containing 100 µl of purified HPV plasmid DNA in TE –buffer with 1mM EDTA and 10 ng/ml of human placenta DNA (Obtained from Dr. Joakim Dillner, WHO HPV LabNet Global Reference Laboratory, Department of Clinical Microbiology, University Hospital, Malmo, Sweden). These reference plasmids were already tested by the reference assay (Linear Array).

Testing of samples:

DNA extraction was done using ethanol precipitation method (Gravitt et al.)(165). The samples were then tested in batches for HPV DNA by polymerase chain reaction (PCR). Extracted DNA was amplified using the L1 primers (HPV specific) and HLA-DQ gene specific primers. The latter was used as an internal control for cell adequacy in the sample. HPV DNA detection was done by gel electrophoresis to ensure optimum conditions of amplification as assessed by HLA-DQ house-keeping gene.

All samples with a positive HLA band in PCR and valid Pap smear and CD4 count report were finally tested by both the assays – the CHUV reverse hybridisation genotyping assay and the Linear Array genotyping assay. The procedure for Linear Array was done as per manufacturer's instructions (Roche Diagnostics, Branchburg, NJ, USA). First round of testing was done by CHUV assay and then all the samples

were tested by the Linear Array. The algorithm for testing the samples is depicted in Figure 1.

I. The Index Test:

PGMY - CHUV Reverse Hybridization assay:

The CHUV assay was developed by the Institut de Microbiology, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland. The assay was designed with the aim to detect HPV DNA based on the PGMY primers and genotype the HPV-positive samples using a reusable probe array (PGMY-CHUV). The assay allows the genotyping of 40 to 80 samples per day and has been used as an adjunct to cytology as well as for the monitoring of treated patients and for epidemiological purposes for 10 years in CHUV, Lausanne. It was developed under a quality assurance program to ensure its reproducibility and accuracy and was optimized in terms of costs ($2.40 per sample). It has been evaluated within the WHO HPV Laboratory Network after technology transfer to member laboratories and was found to be suitable for HPV genotyping with successful participation in proficiency panels established in this network (166).

Brief outline of procedure: The cervical samples were subjected to PCR with PGMY 09/11 primers with HLA-DQ gene as internal control, following DNA extraction using ethanol precipitation. Reactions were analyzed by gel electrophoresis, and all samples hybridized against 37 HPV type-specific probes, covalently bound as an array of parallel lines on a reusable membrane. For hybridization, heat-denatured amplicons were diluted in hybridization buffer and applied on the membrane using a miniblotter

so that they crossed each probe perpendicularly. Hybrids were then revealed by chemiluminescence.

HPV detection using the PGMY09/11 PCR: (130)

Principle: The PGMY primer system amplifies mucosal HPV types, producing a 450 bp amplicon. To confirm adequate DNA extraction and amplification, primers for a cellular target, HLA, producing a 230 bp amplicon were also included. Amplicons were then detected by gel electrophoresis, and genotyped, if positive, using Reverse Hybridisation with the CHUV membrane. Only samples with a positive HLA band by PCR were taken for genotyping by RBH.

I. DNA extraction from Digene STM (using Ethanol precipitation):(165)

A. Materials:

- 7.5 M Ammonium acetate
- 100% Ethanol
- TE buffer: 20mM Tris-HCl + 1mM EDTA [pH = 8.5]
- 20 mg/ml Proteinase K
- 10% Tween -20

B. Procedure:

1. DNA extraction was performed in the 'PCR dirty' room. Work area was prepared, two heat blocks were used, at 65^{o}C and 95^{o}C; microcentrifuge tubes for each sample were labeled.
2. The following were freshly prepared during each run:
   a) Ammonium acetate/Ethanol precipitation solution (10 ml)
      - 8.9 ml – 100% Ethanol (89% final concentration)
      - 1.1 ml – 7.5M Ammonium acetate (0.825 M final concentration)
   b) 10X Digestion buffer
      - 700 µl – TE buffer
      - 100 µl – 10% Tween-20
      - 200 µl – 20 mg/ml Proteinase-K
3. STM samples were thawed and vortexed thoroughly.
4. Fifteen microlitres of 10X digestion buffer was added to each tube followed by 150 µl of the pelleted sample. Pellets were obtained after spinning in a centrifuge at 8000 rpm.
5. These were vortexed to mix and incubated at 65^{o}C for one hour.
6. After incubation at 65^{o}C, samples were spun and incubated at 95^{o}C for 10 minutes.
7. Six hundred microlitres of ethanol precipitation solution was added to each tube, mixed well by inverting the rack 15-20 times and stored at -20^{o}C overnight.
8. After overnight incubation, tubes were kept in a microcentrifuge at 21,000g for 30 minutes at 0^{o}C or at room temperature to pellet DNA.

9. The supernatant was carefully drawn off taking care not to disturb the pellet.
10. Pellets were dried at 55^{o}C for 30 minutes with lids open.
11. DNA was resuspended in 75 µl of TE buffer, vortexed to mix and stored at -20^{o}C until further use (for amplification).

II. Amplification by Polymerase Chain Reaction (PCR):

Principle: Polymerase chain reaction amplifies a specific target region of the template DNA strand. Using suitable primers and cycling conditions the L1 gene of HPV DNA was amplified and identified using the PGMY primers.

A. Preparation of Mastermix: [3.0mM $MgCl_2$]

- Mastermix and reagents were all prepared in the 'clean' room or 'DNA-free' room.

- Prior to starting the preparation, the number of samples being tested and the CHUV ID of the samples were noted in a work logbook. The samples were numbered and accordingly tubes marked.

- A worksheet indicating the position of the samples and controls in the PCR tray was prepared during each run.

Materials	**Volume (in µl for 1X concentration)**
a) Sterile water	- 31.45
b) Buffer II	- 5.0
c) dNTPs	- 0.5
d) $MgCl_2$	- 6.0
e) PGMY09 mix	- 0.8
f) PGMY11 mix	- 0.8
g) HLA – DQ mix	- 0.2
h) AmpliTaq Gold	- 0.25
Total volume =	**45.0**

B. Procedure for amplification (166)**:**

1. DNA extracts were removed from the storage area, brought to room temperature and spun briefly in a microcentrifuge.
2. Negative and positive controls were used with each set of samples.
3. Forty-five microlitres of the Mastermix was distributed in the PCR tubes.
4. Five microlitres of the sample DNA was added into each of the tubes [Final volume = 50 µl] and the tubes transferred to a thermocycler. The thermocyclers used were Veriti ^{TM} Thermal Cycler (Applied Biosystem, Foster City, California, USA) or the GeneAmp® PCR system 9700 (Applied Biosystem, Foster City, California, USA).
5. The target was the HPV L1 gene.

6. The cycling conditions were:
    - Initial denaturation: 95°C for 9 minutes
    - Denaturation: 95°C, 30 sec
    - Annealing: 55°C, 1 min 30 sec
    - Extension: 72°C for 2 min

    } X 45 Cycles

    - Final extension: 72°C for 5 min
7. The expected amplification product was 450 base pairs long.

C. Post amplification DNA detection by Gel Electrophoresis:

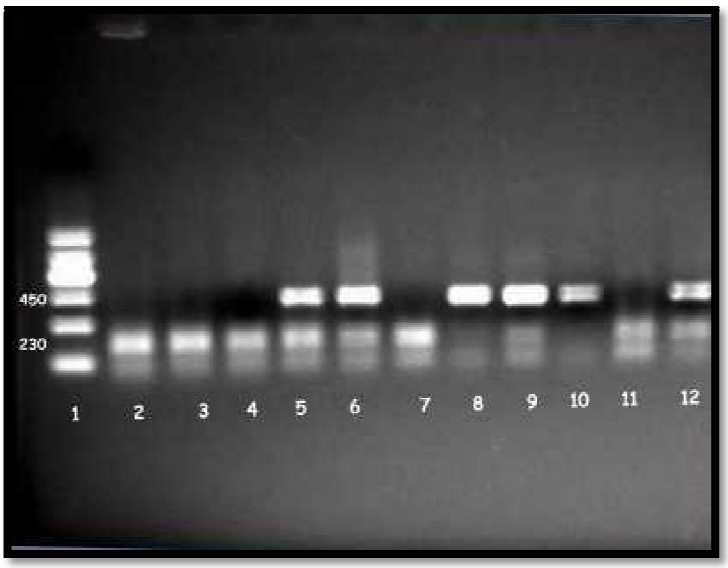
HPV DNA detection was done by Gel Electrophoresis.

1. Hundred milliliters of 1% agarose gel in 0.5X TBE was prepared and boiled in a microwave until fully dissolved. After cooling, 10 µl of Ethidium Bromide was added, the gel poured into the gel electrophoresis apparatus, comb placed at one end and gel allowed to set.
2. Once the gel cooled and set, 0.5X TBE was added to cover the gel by 1cm.
3. Ten microlitres of each amplicon was subjected to electrophoresis along with 2 µL of 6X loading dye bromophenol blue in freshly prepared gel of 1 % agarose.
4. Test samples, positive and negative controls were loaded into appropriate wells.
5. Molecular ladder (DNA Marker-C, Bio Basic Inc.Canada) used was 100-1200 bp long.
6. Electrophoresis was done at 15 Watts constant power for one hour.
7. Gel documentation was done using the BioRad Gel Documentation system (BioRad Laboratories, Hercules, USA).

D. Interpretation of gel electrophoresis results:

The adequacy of each of the cervical samples was evaluated by the presence of a HLA band at the level of 230 bp, which indicated the adequate number of cells in the specimen. The isolate was considered positive for HPV L1 gene when a band was visible at the level of the positive control band at 450 bp. Samples which showed a band only at 230 bp and not at 450 bp were considered negative. It indicated that these samples did not contain HPV and therefore did not produce any visible band.

Figure: Documentation under UV light following amplification



Lane 1: Molecular ladder

Lane 2-11: Clinical samples (Cervical brush samples)

Lane 12: Positive control – Caski (HPV16) – shows positive HLA band (230 bp) and HPV band (450 bp)

Lanes 2, 3, 4, 7, 11: Clinical samples positive for HLA band (230 bp) but negative for HPV band (450 bp)

Lanes 5, 6, 9: Clinical samples positive for HLA band (230 bp) and HPV band (450 bp)

Lanes 8 and 10: Clinical samples negative for HLA band (230 bp) and positive for HPV band (450 bp)

III. HPV genotyping by reverse blot hybridisation and chemiluminescent detection:

A. Sample application and hybridization(166)**:**

Principle: After heat denaturation in a low salt buffer, the biotinylated HPV amplicons are hybridised to a parallel array of HPV type-specific probes covalently bound to a negatively – charged nylon membrane. After washing, the hybrids are revealed by chemiluminescence on an autoradiography film. All 50 clinical samples and the 43 WHO reference samples were tested by RBH.

The procedure is outlined below:

1. At start of the procedure, the hybridization oven - HybriLinker™ Hybridization Oven (Upland, CA, USA) was turned on and the shaker bath set to 50^{o}C. The hybridization buffers were pre-heated in the water bath before use.
2. Ten microlitres of the amplicon was denatured in 110 µl of PCR grade water at 95^{o}C for five minutes. Immediately afterwards, the tubes were cooled by placing them on ice.
3. One hundred and ten microlitres of 8X SSPE (0.4% SDS), pre-heated to 50^{o}C, was added and mixed well.
4. The miniblotter was assembled and the membrane placed, such that it covered the bottom of the channels, with the probes facing upwards. Membrane was placed so that the ink lines were perpendicular to the channels. All the wells were aspirated; 2X SSPE added sequentially to all wells and re-aspirated before sample application.

5. One hundred and fifty microlitres of the sample was added through the top row of wells to alternating channels, with 2X SSPE (0.1% SDS) in the intervening channels. Positive and negative controls were used with every run.
6. A scotch tape was placed on the wells to limit evaporation.
7. The miniblotter with the membrane was transferred to a hybridization oven and kept at 50^{o}C for 90 minutes.
8. After hybridization, the channels were vacuum aspirated, the miniblotter disassembled and the membrane placed in 250 ml of 2X SSPE (0.5% SDS) at 50^{o}C.
9. The membrane was washed at 50^{o}C for 10 minutes with gentle agitation.
10. Five microlitres of streptavidin peroxidase diluted in 2X SSPE (0.5% SDS) was added and the membrane incubated at 42^{o}C for 45 minutes.
11. The membrane was then washed twice in 250 ml 2X SSPE (0.5% SDS) for 10 minutes each; and rinsed twice in 250 ml of 2X SSPE at room temperature for 5 minutes each.
12. Excess liquid was blotted dry from the membrane and it was placed face down (nucleic acids face down) in 4 ml of ECL detection solution for 2 minutes, such that the liquid spread evenly under the membrane.
13. After blotting the excess liquid dry from the membrane, it was placed between two A5 transparencies and transported in an x-ray cassette.
14. Chemiluminescent detection was done in the department of Radiology, CMC, Vellore in the dark room – Here, an autoradiography film was placed on top of the

assembly in an exposure cassette and the film exposed for 1 hour. Subsequently, the film was developed and the readings taken.

15. Sample IDs were indicated next to the HLA spot of each sample.

B. Interpretation of the reverse hybridization assay: HPV types were assigned for each sample by visual inspection of the developed film against light, using the CHUV array scheme as guide. Positives were identified by signal strength superior to that of local background, square shape of the spot and its expected matrix position. A sample was considered to have a HPV type only if the HLA spot was also positive. Multiple types were detected if multiple spots occurred along the same lane as the HLA spot. If a sample had only HPV spot(s) and no HLA spot, the result was considered as invalid and excluded from analysis. Very faint signals which did not comply with the definition of a positive were ignored and taken as negative. Evaluation was done by two individuals independently to avoid errors due to misreading.

II. The Reference test:

Linear Array genotyping test(Roche diagnostics, Branchburg, NJ USA):

Principle: The Linear Array HPV genotyping test is a CE-IVD approved kit for genotyping 37 high and low risk HPV types. The test is based on four major processes: specimen preparation, PCR amplification of target DNA using HPV PGMY primers, hybridization of amplified products to oligonucleotide probes and detection of the probe-bound amplified products by colorimetric determination.

The materials for testing were provided in the LA kit and the reagents were prepared based on manufacturer's instructions.

A. DNA Extraction and PCR amplification:

1. DNA extraction was done by ethanol precipitation as per the procedure previously described (165).
2. Pre-amplification Reagent preparation – this was performed the following day in the 'DNA-free' room.
3. Prior to starting the preparation, the number of samples being tested and the CHUV ID of the samples were noted in a work logbook. The samples were numbered and accordingly tubes marked.
4. A worksheet indicating the position of the samples and controls in the PCR tray was done during each run.
5. The Mastermix was prepared by adding 125 µl of HPV Mg2+ to one entire vial of HPV MMX, mixed by inverting the tube 10-15 times. The remaining HPV Mg2+ is discarded.
6. Fifty microlitres of working Mastermix was added to each of the reaction tubes.
7. The tray with the tubes was moved to the 'PCR dirty' room.
8. Positive and negative controls were used with each run.
9. Fifty microlitres of the sample was added to each reaction tube.

10. The thermocyclers used for amplification were Veriti ™ Thermal Cycler (Applied Biosystem, Foster City, California, USA) or the GeneAmp® PCR system 9700 (Applied Biosystem, Foster City, California, USA).

11. The cycling conditions were:

    - Initial denaturation: 50°C for 2 min and 95°C for 9 min
    - Denaturation: 95°C, 30 sec
    - Annealing: 55°C, 1 min
    - Extension: 72°C for 1 min

    } X 40 Cycles

    - Final extension: 72°C for 5 min

12. The expected amplification product was 450 base pairs long.

B. Post amplification HPV detection and genotyping:

1. The amplified products were removed and taken for hybridization within 4 hours of PCR, and mixed well by pipetting.
2. Waterbath was pre-heated to 53°C and set at a shaking speed of approximately 60 RPM.
3. All the reagents were brought to room temperature. The Working Hybridization buffer, Ambient Wash buffer, Stringent Wash Buffer and Citrate buffer were all prepared as per kit instructions and pre-heated in the waterbath.
4. The strips for hybridization were removed from the pouch and handled using clean forceps, marked such that one strip was assigned to only one sample (CHUV ID) and placed in the 24-well tray.

5. Pre-warmed hybridization buffer was added to each well with the strip.
6. Seventy-five microlitres of the denatured amplicon was added to the appropriate well and the tray rocked gently between each addition. The tray was covered with the lid and placed in the waterbath for hybridization for 30 minutes at 60 RPM.
7. Meanwhile, the working conjugate was prepared by adding 15 µl of SA-HRP to 5 ml of Working Ambient Buffer for each strip being tested and mixed well.
8. After hybridization, the tray was removed, the wells were vacuum aspirated and 4 ml of Working Ambient Buffer added to each well and aspirated immediately.
9. Four millilitres of pre-warmed Working Stringent Wash Buffer was then added to the strips and incubated for 15 minutes at 60 RPM in the waterbath.
10. After incubation, the wells are vacuum aspirated and 4 ml of Working Conjugate added to the wells. The tray was then incubated at room temperature (15-30^{o}C) for 30 minutes at 60 RPM.
11. The wells were then vacuum aspirated, 4 ml of the Working Ambient Buffer added and incubated for 10 minutes at room temperature at 60 RPM. This step was repeated again.
12. After vacuum aspiration of the wells, 4 ml of the Working Citrate buffer was added and incubated for 5 minutes at room temperature at 60 RPM.
13. The wells were vacuum aspirated and 4 ml of the Working Substrate is added to each well, incubated for 5 minutes at room temperature at 60 RPM.
14. The wells were vacuum aspirated and 4 ml of deionised water added to each well containing the strip. The strips were then removed using forceps and placed on clean and dry surface and allowed to air dry.

15. Readings were taken within one hour of drying and confirmed again the following day.

Interpretation of LA results:

For interpretation, the Linear Array Genotyping Test Reference guide was placed over each strip and genotype readings taken. The low and high β-globin bands had to be positive for the sample. The results of the positive and negative controls were taken first to confirm that the run was valid and that there was no cross-contamination between wells. Evaluation was done by two of individuals independently to avoid errors due to misreading.

The interpretation of the Linear Array strips was done as follows:

HPV Result	β-globin Low result	β-globin High result	Interpretation
-	-	-	**Invalid**
-	-	+	**Invalid**
-	+	+	**HPV DNA not detected**
+	-	-	**HPV DNA detected (Report genotype)**
+	-	+	**HPV DNA detected (Report genotype)**
+	+	+	**HPV DNA detected (Report genotype)**

Statistical analysis: The accuracy indices of the CHUV assay in comparison to the Linear Array – sensitivity, specificity, positive predictive value and negative predictive were calculated with a 95% confidence interval. Level of agreement or kappa value between the two assays was also calculated. All data generated in the study were analyzed using the SPSS software - version 16.0.

ALGORITHM OF TESTING SAMPLES

50 clinical samples + 43 WHO reference samples

(stored at -20^{o}C)

Thawed before testing

↓

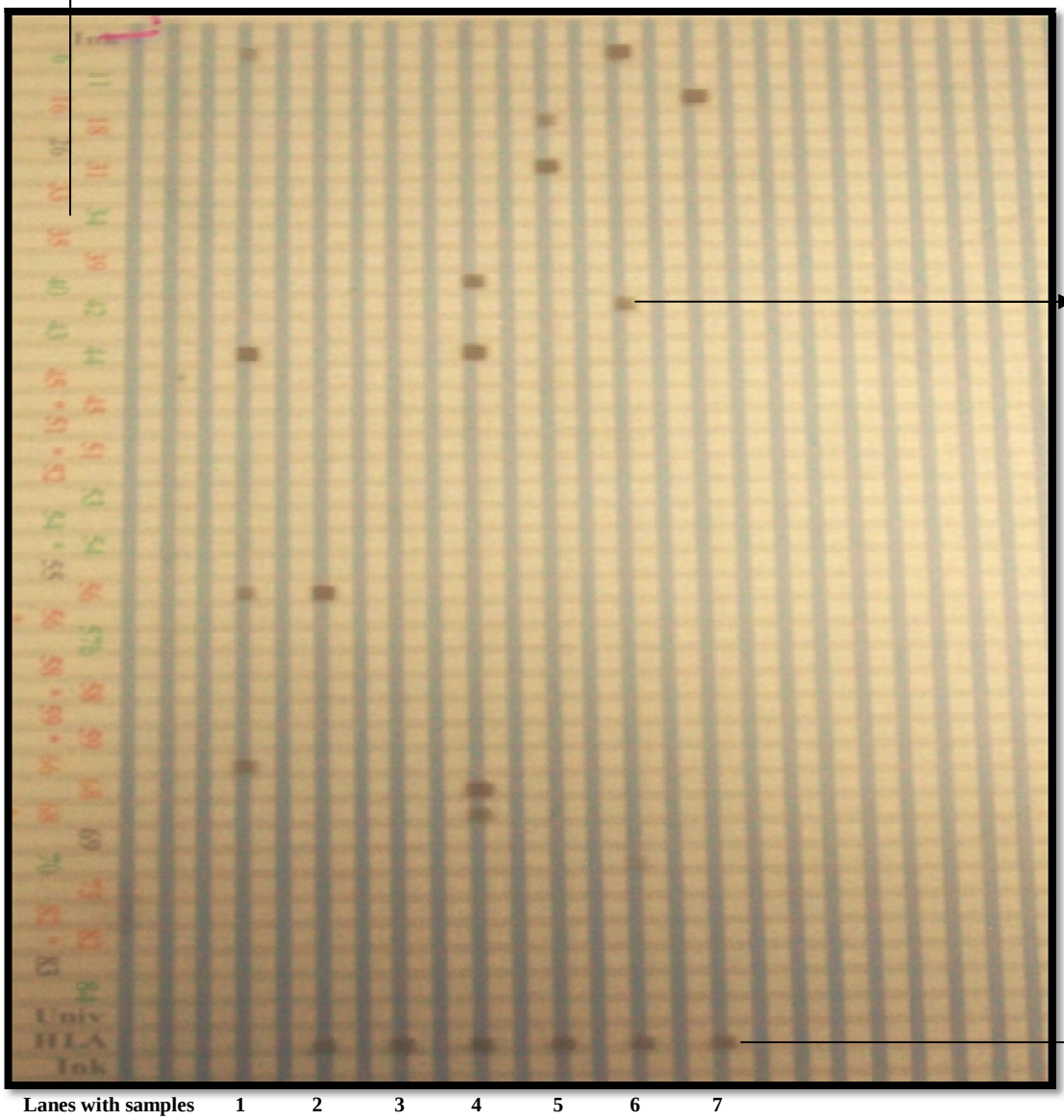
DNA Extraction by Ethanol precipitation method

(Clinical samples only as WHO reference samples consist of purified plasmids)

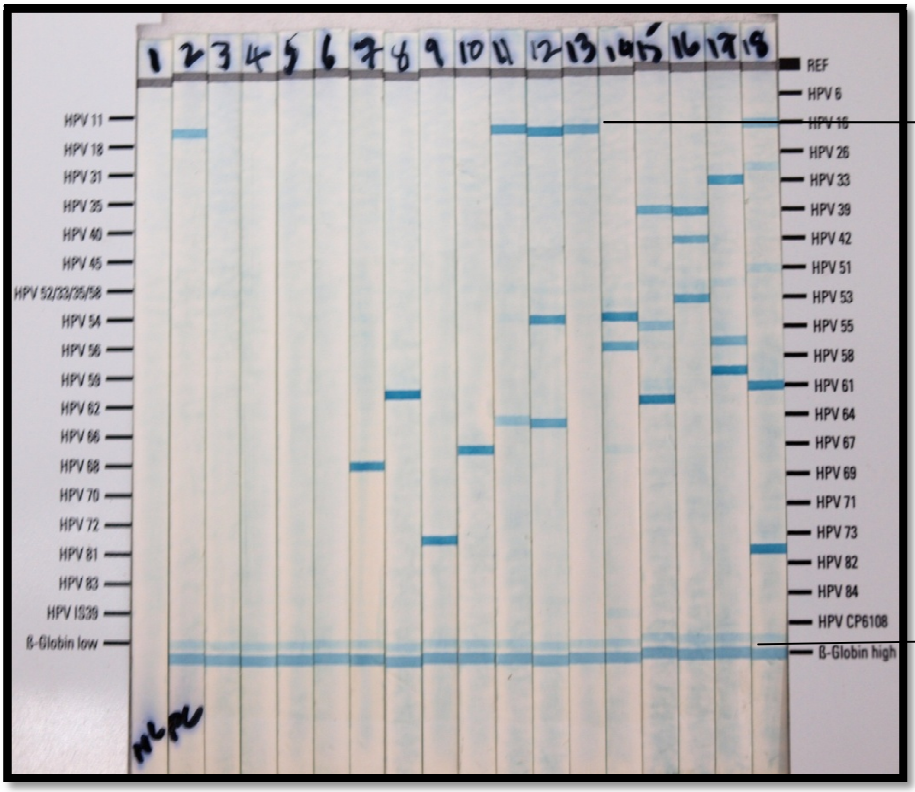
CHUV assay	**Linear Array**
Biotinylated PGMY09/11 primers & HLA-DQ as internal control	PGMY09/11 primers & β-globin gene as internal control
↓ Amplification	↓ Amplification
Gel Electrophoresis	
↓	
Denaturation	Denaturation
↓	↓
Hybridisation to a membrane with HPV type-specific probes	Hybridisation to a strip with HPV type-specific probes
↓	↓
Detection of specific HPV genotypes by Chemiluminescence (In a dark room)	Visual detection of specific genotype on strips – positives show coloured band against the particular genotype

CHUV assay: Interpretation

Array scheme showing genotypes



Linear Array: Interpretation



Positive band

β-globin band – Control band

RESULTS

A. Demographic data:

Majority of the women were from Tamil Nadu (38/50, 76%), the rest from states of Andhra Pradesh (7/50, 14%), Karnataka (2/50, 4%), Orissa (2/50, 4%) and Maharashtra (1/50, 2%). The ages of the patients ranged from 21- 40 with the median age being 32.2 years. All were married women, majority having only one sexual partner (90%) and 5 of them (10%) having two or more sexual partners. The age of onset of sexual activity ranged from 15 – 29 years and mean age was 19.9 years; the age of onset of sexual activity less than 18 years in 36% women (18/50) and more than 18 years in 64% (32/50) women. The median parity for the study group was 1.46; 10 out of 50 women had no children (20%), 16 women had 1 child (32%) and 24 women had 2 or more children (48%). Most women were newly diagnosed HIV cases, the duration of HIV diagnosis being less than 5 years for 41 (82%) patients and more than 5 years for 9 (18%) patients. The distribution of patients according to the WHO clinical staging of disease was Stage I - 44 women (88%), Stage III – 1 woman (2%), and Stage IV – 5 women (10%). CD4 counts were below 350 for 17 patients (34%) and more than 350 for 33 patients (66%). Pap smear reports of majority of patients were normal (43/50, 86%), LSIL and HSIL being reported in 1 patient each (4%). Other STIs were diagnosed in only 4 patients (8%), 2 women diagnosed with syphilis and 2 others hepatitis B (probable sexual transmission as there was no history of a blood transfusion). Partner HIV-status was positive for 43 patients (86%), negative for 4 patients (8%) and not known/ undiagnosed for 3 patients (6%). The demographic data are depicted on pages 74 and 75.

B. Comparison to Linear Array:

The results of PGMY - CHUV were compared to another PCR-reverse hybridisation assay which is a commercially available and CE marked assay- the Linear Array (LA). For comparison, 50 clinical samples were tested with both the assays. In addition, 43 WHO samples that were previously genotyped by LA were also evaluated with PGMY - CHUV.

B.1 HPV positivity by PCR only: All samples were tested with positive and negative controls during each run. Interpretation of results was done as mentioned earlier. In the PGMY-CHUV assay, HLA band was used an internal control to look for cell adequacy in the samples. The first 50 clinical samples that were positive for HLA by PCR and who had adequate clinical information i.e, CD4 count and Pap smear results were selected. Of these selected samples, 4 out the 50 clinical samples gave a negative β-globin band in the Linear Array (LA). Among the 50 clinical samples, only 21 (42%) were positive for HPV by the PGMY-CHUV. This was prior to performing the reverse hybridisation (RBH).

Of the 43 WHO samples, 42 were known HPV positives and 1 sample was a known negative. The PGMY-CHUV PCR gave a positive result for 21 samples (48%) which showed a visible band for L1 gene of HPV.

B.2 HPV positivity by CHUV - RBH:

The rate of detection of HPV improved when the RBH results were subsequently included. HPV genotype(s) were detected in 27 out of 46 (59%) clinical samples by CHUV – RBH compared to 22 out of 46 (48%) detected by Linear Array. Only 9

samples were consistently reported positive by both the assays, the other positives being discordant (exclusively 10 by CHUV and 5 by LA).

As four clinical samples lacked a β-globin and HPV band in the Linear Array, they were interpreted as invalid and excluded from further analysis.

Similarly, genotypes were detected in 36 (84%) of the 43 WHO reference samples by PGMY-CHUV RBH as compared to 39 of 43 samples (91%) detected by Linear Array. Overall, when both sets of samples (clinical samples + WHO reference panel) were combined, HPV was detected in 63/89 samples (71%) by the CHUV assay and in 61/89 samples (69%) by the Linear Array.

Refer Figure 1.

C. Genotyping results:

C.1 CHUV assay genotyping results:

i) **Clinical Samples**: Single HPV infection was seen in 15 of the 27 (56%) positive clinical samples; multiple HPV infection seen in additional 12 samples (44%). Two samples were positive for HPV DNA only by PCR and no genotype was detected in RBH. Totally, 16 different array-specific genotypes were detected. The highest number of genotypes in a single sample was four (1 sample – HPV16, HPV42, HPV59, HPV66); 2 samples had three genotypes, 9 samples had two genotypes and 15 samples had only a single HPV type**.** The most common genotype was HPV16 (9 samples), followed by HPV31 (5 samples), HPV51 and HPV59 (4 samples each), HPV6 and HPV42 (3 samples), HPV54, HPV55, HPV56, HPV66 (2

samples each), HPV18, HPV39, HPV40, HPV44, HPV45 and HPV70 (1 sample each) in descending order. High-risk HPV types detected were HPV16, HPV18, HPV31, HPV39, HPV45, HPV51, HPV56 and HPV66. Low risk HPV types HPV6, HPV40, HPV42 and HPV44 were seen, HPV42 being the most common.

ii) **WHO reference samples:** Single HPV type was detected in 29 of the 42 positive samples (71%), and multiple types in 7 samples (14%). The total number of different HPV types recognized was 16. The maximum genotypes detected in a single sample was five which had types HPV35, HPV39, HPV59, HPV66 and HPV68; four HPV types were seen in 3 samples, three types in 2 samples and two types in 1 sample. The most common genotypes detected were HPV11, HPV45, HPV51, HPV58 and HPV59 (4 samples each). The other types detected were HPV6, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV52, HPV66 and HPV68 (3 samples each), and HPV56 in 1 sample. High risk types detected were HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV66 and HPV68. Among the low risk types only HPV6 and HPV11 were seen. HPV types not detected, in the 6 known positive WHO reference samples, by the CHUV assay were HPV6 (1 sample), HPV16 (1 sample), HPV56 (2 samples) and HPV68a (2 samples). HPV68 was missed by CHUV assay in 5 samples.

C.2 Linear Array results:

i) **Clinical samples:** Single HPV type was detected in 8/22 positive samples (36%) and multiple types in 14/22 samples (63%). The highest number of types detected was four HPV types in 4 samples each; infection with three types was seen in 2 samples and with two types seen in 8 samples. The total number of array – specific genotypes detected were 19 HPV types, the commonest being HPV16 (11 samples), followed by HPV51 and HPV59 (5 samples each); HPV6, HPV31, HPV42, HPV45, HPV54, HPV62, HPV66, HPV81 and HPV-CP6108 (2 samples each); types HPV18, HPV39, HPV40, HPV56, HPV61, HPV68 and HPV70 (1 sample each). High risk types detected include HPV16, HPV18, HPV31, HPV39, HPV45, HPV51, HPV56, HPV59, HPV66 and HPV68; low-risk types HPV6, HPV40, HPV42, HPV54, HPV61, HPV70, HPV81 and HPV-CP6108.

ii) **WHO reference samples:** The WHO samples were a set of 42 known HPV positives and 1 known HPV negative sample. These were previously genotyped samples and the results are as follows:

Single HPV type was detected in 31 out of 42 positives (74%) and multiple types in 8 /42 samples (19%). The highest number of genotypes in a single sample was five, being detected in 2 samples each; followed by four genotypes in 4 samples and three genotypes in 2 samples. The total number of genotypes detected was sixteen and include HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV58,

HPV59, HPV66 and HPV68, all of which were detected in 4 samples each; HPV56 was detected in 3 samples. The high risk types detected were HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66 and HPV68; low risk types HPV6 and HPV11. LA missed HPV types 68 (4 samples) and HPV56 in 1 sample.

C.3 Comparison of PGMY – CHUV assay with the Linear Array genotyping assay (Table 2):

i) **HPV detection:** The overall rate of detection of HPV was higher with the PGMY –CHUV assay when compared to LA results. Of the clinical samples, 29 (63%) were positive for HPV versus 21 (45%) samples which were positive by LA. Among the WHO samples, 36/43 (84%) were positive by CHUV compared to 39/43 (91%) positive by LA. Combined the rate of detection was 75% by CHUV vs. 70 % by LA.

Refer Figure 1.

ii) **Single vs. Multiple HPV infection:** CHUV reported 66% and 71% single HPV types among the positive clinical and WHO samples respectively, while LA reported 36% and 72% single HPV types in the same panels. Multiple HPV types were detected better by LA, 63% and 19% in clinical and WHO samples respectively, when compared to 44% and 14% by the CHUV assay. Overall, the rates of detection of single and multiple HPV types by the two assays

respectively were – 70% and 29% (CHUV) and 61% and 34% (LA). Refer Figure 2.

iii) **Genotypes:** Both assays picked up an array of 16 genotypes. Among the clinical samples, Linear Array identified four additional genotypes compared to CHUV: HPV61, HPV62, HPV81 and HPV-CP6108 whereas types HPV44 (1 sample) and HPV55 (2 samples) were detected only by the CHUV assay. The CHUV assay was able to pick up an additional HPV-56 in a clinical sample. The HPV types identified in WHO reference samples by both the assays were similar. However, both the assays missed HPV types 68 (CHUV- 5 samples; LA- 4 samples) and 56 (1 sample) in the WHO reference panel.

Figures 3 & 4 depict the number of genotypes detected by both the assays.

Figure 5 shows the range of genotypes detected by the two assays.

iv) **High-risk types detected:** Linear array identified high-risk type HPV16 in 11 clinical samples compared to in 9 samples by CHUV. The other high-risk types detected was similar, type HPV31 being detected more frequently by CHUV (5 clinical samples) and HPV51 and HPV59 (5 samples) by LA. Types that were recurrently missed by the CHUV assay were HPV16 (3 samples- 2 clinical+1WHO sample) and HPV59 (3 samples).

Figures 6 and 7 depict the high-risk HPV types detected by both the assays in the clinical panel and the WHO panel.

v) **Correlation with Pap smear and CD4 counts:** In the two patients with precursor lesions (LSIL and HSIL), HPV was detected by both CHUV and LA. In the patient with LSIL, CHUV detected types HPV18 and HPV31 while LA

detected types HPV31 and HPV81. Similarly in the patient with HSIL, CHUV detected types HPV16, HPV42, HPV59 and HPV66 while LA detected HPV16, HPV59, HPV66 and HPV-CP6108. Type HPV-CP6108 is not included in the CHUV genotype panel.

The CD4 counts of the two patients with LSIL and HSIL was 394 cells/μl and 215 cells/ μl respectively.

vi) **Accuracy indices:** With the Linear Array as the reference standard, the sensitivity of the CHUV assay was 77% and 92% respectively for the clinical and WHO samples. The specificity was much lower (50%) for the clinical samples, however, it was nearly 100% for the WHO samples. Overall, the sensitivity improved for the combined samples being 91%; specificity still remained low at 52%. The accuracy indices of the CHUV assay are depicted in Table 1.

vii) **Level of agreement:** The level of agreement between the two assays ranged from 0.26 (poor agreement) for the clinical samples to 1.000 (perfect agreement) for the WHO samples. On the whole, for combined samples, there was moderate agreement of 0.47 between the CHUV and Linear Array assays. The assays showed moderate agreement for detection of high risk types HPV-16 and 18.

viii) **Cost evaluation:** Taking into account the cost of DNA extraction, PCR and genotyping with RBH, the cost of the CHUV assay for one sample was

estimated to be Rs.1030. The cost of testing with the Linear Array per sample is Rs.4165.

The differences between the two assays in the testing of clinical samples depicted in Table 2.

Comparison of PGMY – CHUV assay with the WHO reference panel:

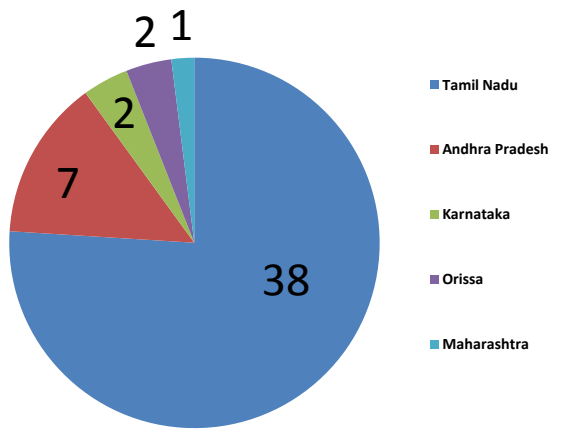
Features	CHUV assay	WHO Reference panel (42 positives + 1 negative)
HPV Positivity	36/42 (86%)	42 positives
Single HPV types/sample	29/34 (85%)	34
Multiple HPV types/sample	7/8 (88%)	8

i) **Genotypes:** The CHUV assay was able to detect the entire range of genotypes in the WHO reference panel. HPV genotypes in most of the WHO reference samples of low concentration including high-risk HPV types (such as HPV-16) were also detected. Three samples with low concentrations of 5 IU and 50 IU gave a negative result.

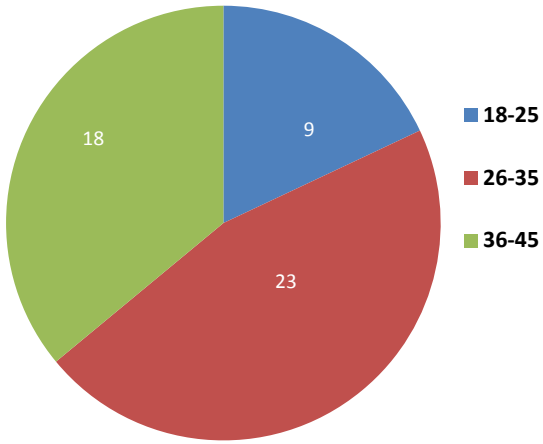
ii) **Accuracy indices** – When the results of CHUV assay were compared to the WHO reference panel results, the sensitivity was around 86% and specificity was 100%. The negative predictive value and positive predictive value were not calculated as the reference panel consisted of 42 positives and only 1 negative. The level of agreement was 1.00 (perfect agreement).

I. Demographic data:

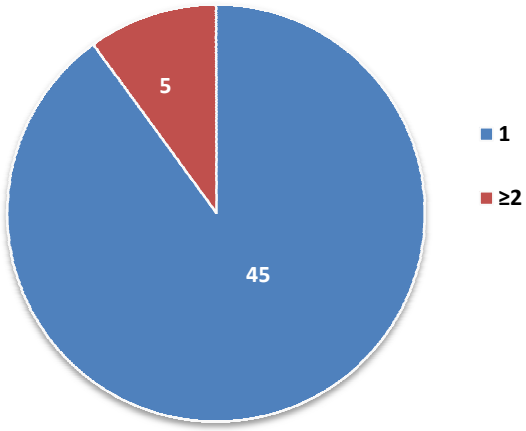
1.Place



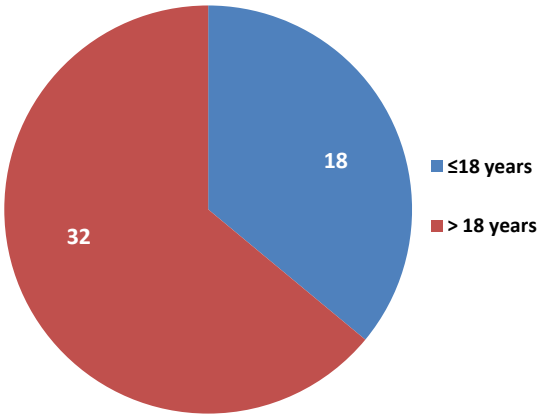
2. Age



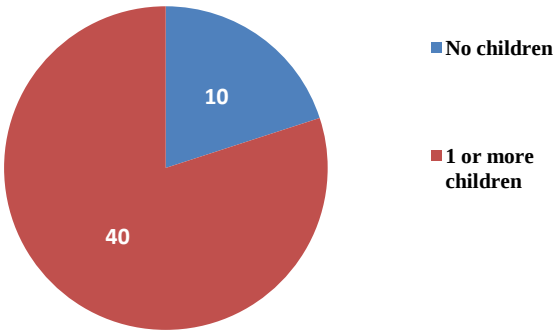
3. No.of sexual partners



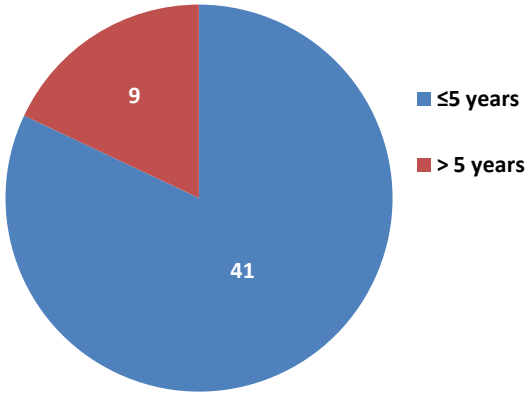
4.Onset of sexual activity



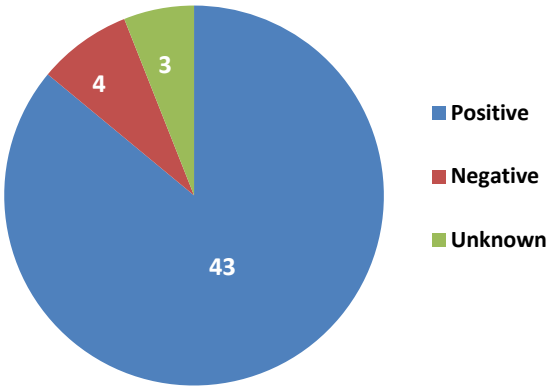
5. Parity



6. Duration of HIV infection



7. Partner HIV status



8.Other STIs

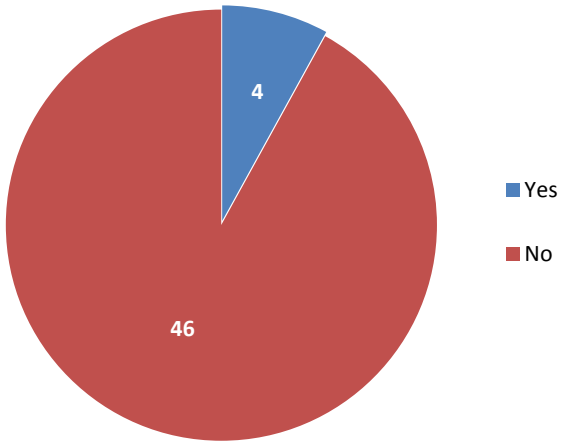


Table 1: Accuracy indices of CHUV assay

Accuracy indices	Clinical samples	WHO samples	Combined (Clinical + WHO samples)
Sensitivity	**77%**	**92%**	**91%**
Specificity	**50%**	**100%**	**52%**
Positive predictive value	**59%**	**-***	**82%**
Negative predictive value	**71%**	**-***	**72%**

***** NPV and PPV not calculated for WHO samples as the reference panel consisted of 42 positives and only 1 negative.

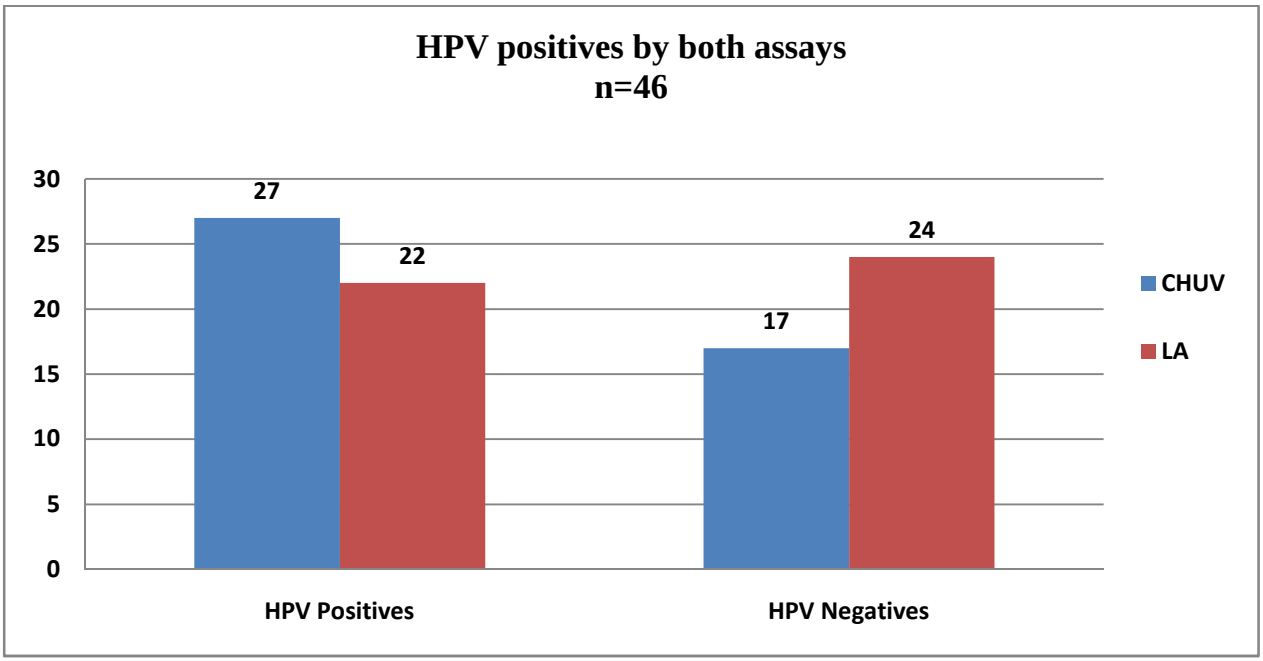
Table 2: Comparison of the CHUV assay with LA for clinical samples

Category	CHUV assay	Linear Array
Single infection	56%	36%
Multiple infection	44%	63%
Genotypes detected	Similar to LA except for 4 types that are not incorporated in the assay	Four additional types detected – HPV types 61, 62, 81 and CP6108;
HPV -16	9 samples	11 samples
Other high-risk HPV types	Similar HPV-31 detected better	Similar HPV-51 and -59 detected better
Capacity of testing samples per run of assay	22 samples (As negative controls are used in intervening lanes)	46 samples
Cost evaluation	Rs.1030 per sample	Rs.4165 per sample

II. Results of the CHUV assay and Linear Array:

Figure 1:

A. HPV positivity by both assays – Clinical samples (PCR + RBH results)



Note: 2 samples in the CHUV assay – were positive only by PCR but no genotype was detected in RBH

B. HPV positivity by both assays – WHO reference samples (PCR+RBH results)

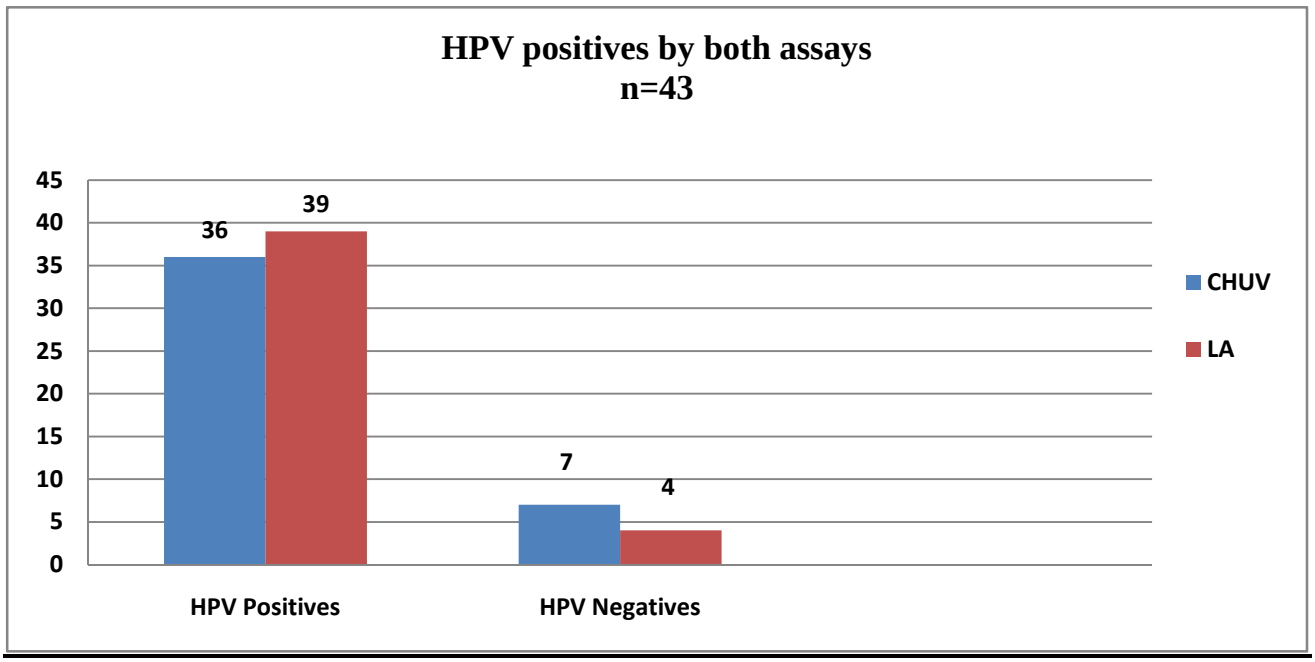


Figure 2: Single vs. Multiple infections detected in clinical samples

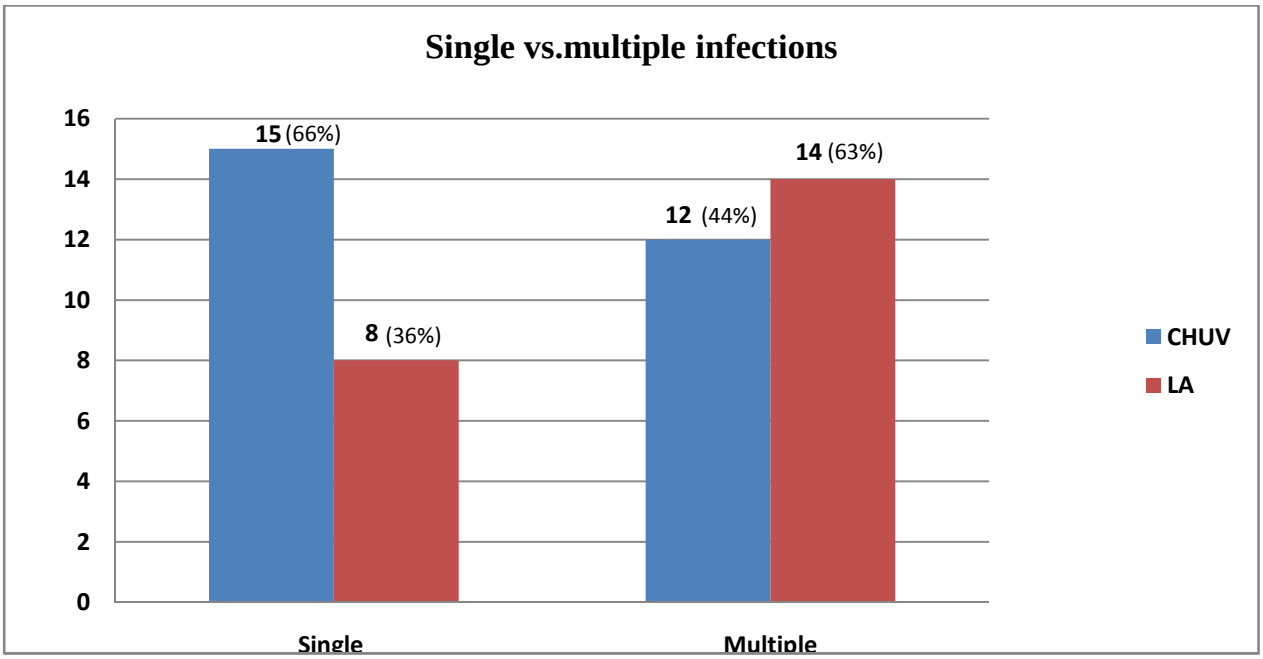


Figure 3: Showing number of mono-infections and multiple infections detected by both assays in clinical samples

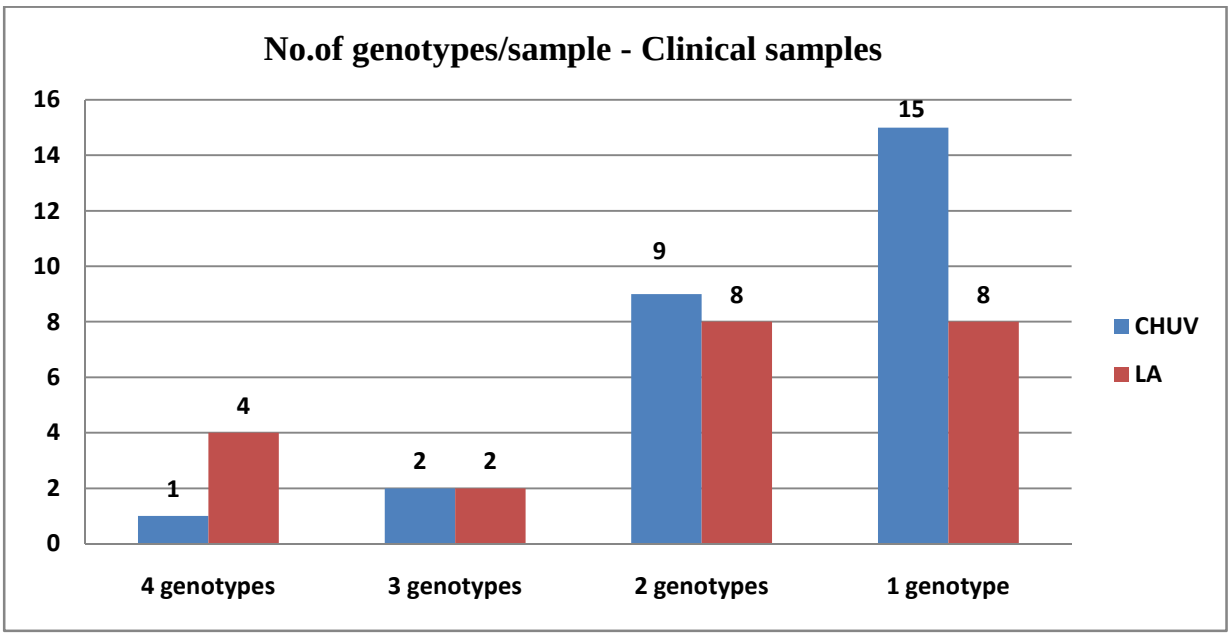


Figure 4: Showing the number of genotypes detected in WHO samples

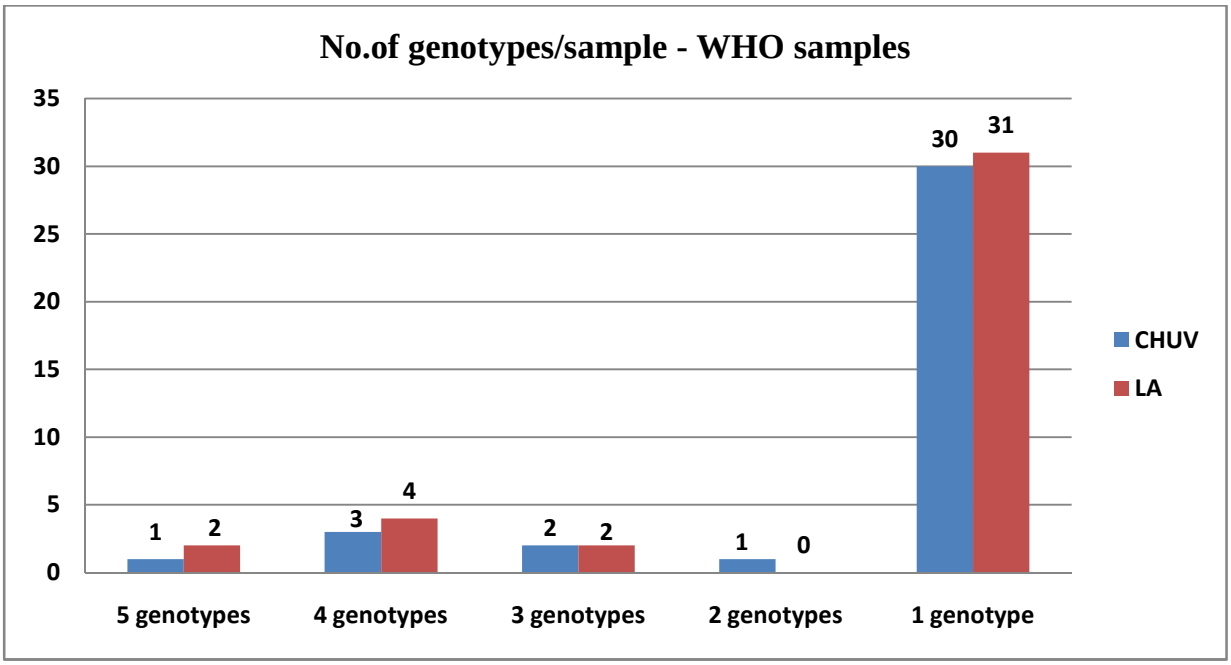


Figure 5: The range of genotypes detected by CHUV assay in clinical samples in comparison with LA results

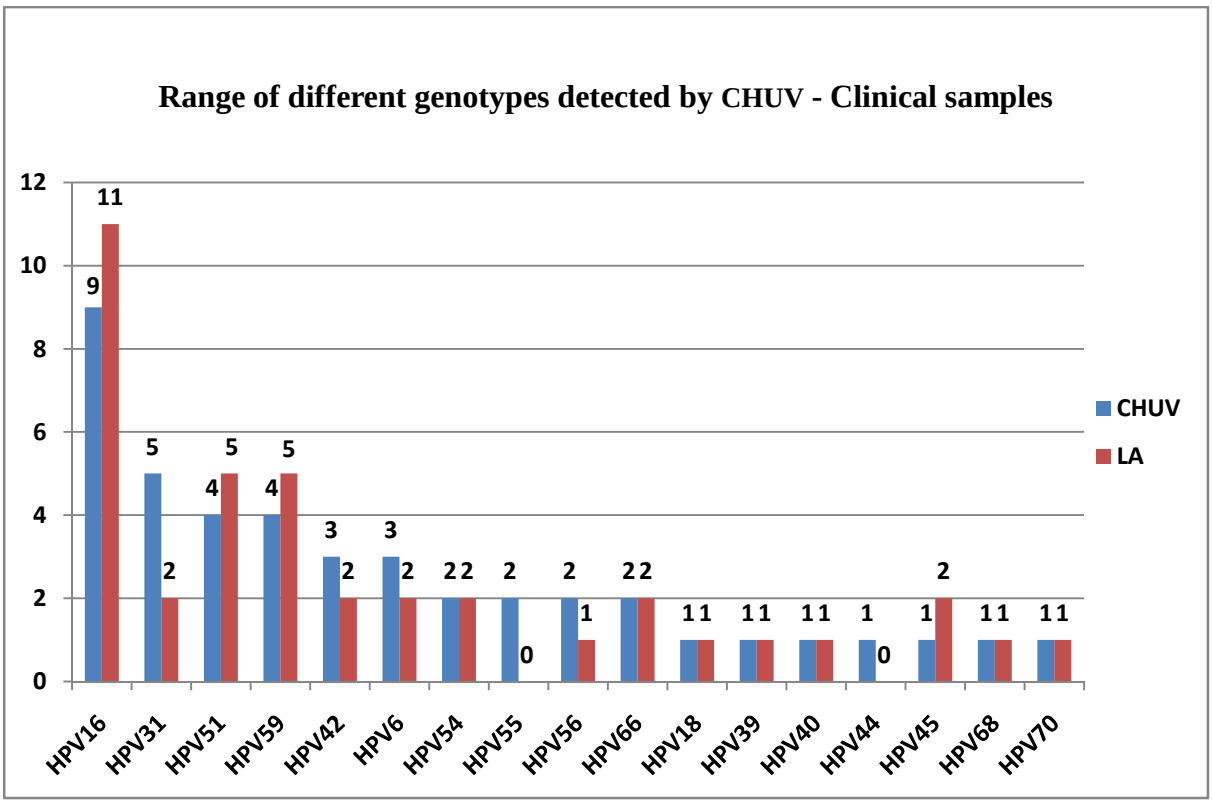


Figure 6: High-risk HPV types detected in clinical samples

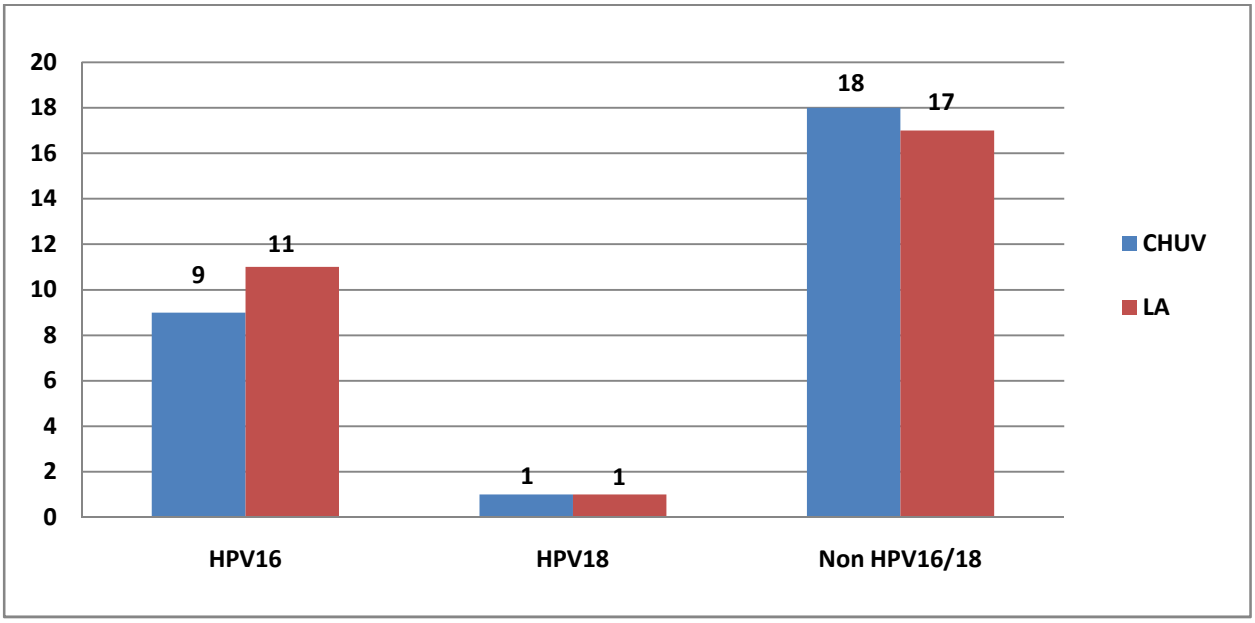
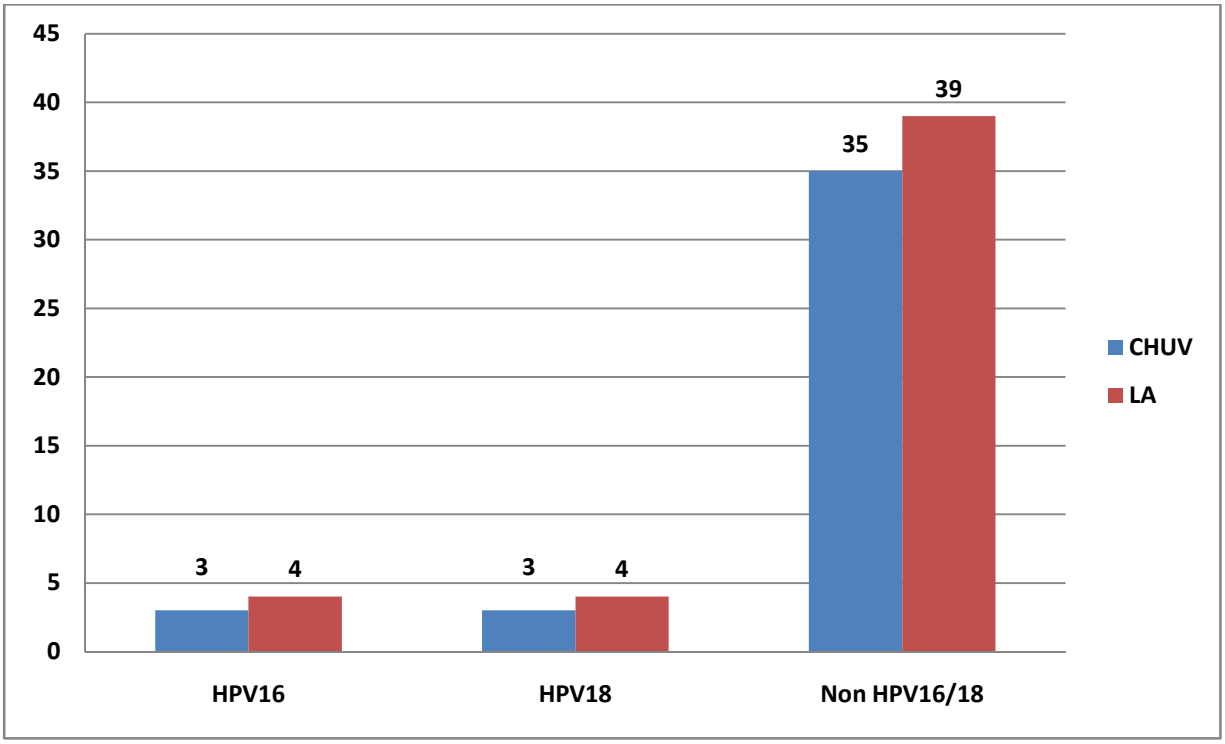


Figure 7:High-risk types detected by both the assays in WHO reference samples



Discussion

Cervical cancer is the most common cancer in women in India. The human papillomavirus is now recognized to be a necessary cause for the development of cervical cancer (1). HIV –infected women tend to have more persistent HPV infection and infection with diverse, multiple HPV types (8). Cervical cancer is also an AIDS-defining illness (167). HPV DNA testing is now being used as an adjunct to cervical cytology for screening and diagnosis of cervical cancer and its precursors. Many PCR-based methods for HPV detection and genotyping are available, but they are expensive. In a developing country like India, with a high burden of cervical cancer, there is a need for a low-cost, sensitive, robust test for HPV detection and genotyping which can be used for screening as well as diagnosis of cervical cancer in women.

We evaluated an in-house reverse hybridization assay (CHUV assay), based on PGMY primers and a reusable probe array, in comparison to the licensed (CE-IVD) Linear Array (Roche Diagnostics) for HPV detection and genotyping. Our study samples included cervical brush samples from 50 asymptomatic HIV-infected women and 43 coded WHO reference HPV plasmids (Obtained from Dr. Joakim Dillner, WHO HPV LabNet Global Reference Laboratory, Department of Clinical Microbiology, University Hospital, Malmo, Sweden). Samples were included from HIV-infected women as the HPV prevalence is higher in this group, and to determine the ability of the assay to detect the multiple HPV types as this category of patients are known to harbour multiple types (8). In addition, WHO panel of coded samples were included to validate the assay. These reference samples were already tested by the

reference assay (Linear Array). Our study showed moderate agreement between the two assays for HPV genotyping.

The PGMY – CHUV assay was developed by the Institut de Microbiology, CHUV, Lausanne, Switzerland. Current guidelines do not validate the PGMY –CHUV assay for primary screening of cervical cancer (168), therefore, it was designed as an adjunct to cytology to detect HPV DNA based on the PGMY primers and to genotype the HPV-positive samples using a reusable probe array (PGMY-CHUV) (166). The assay has been evaluated for genotyping HPV for the past 10 years in the CHUV Institute, Switzerland and within the WHO HPV Laboratory Network and found to be suitable for HPV genotyping with successful participation in proficiency panels established in this network (169). The assay can also be potentially used for epidemiological purposes related to vaccine monitoring.

As a reference standard, the commercial CE-IVD approved Linear Array (LA) was used to evaluate the proficiency of the PGMY – CHUV assay. The Linear Array also uses PGMY primers for HPV DNA amplification and a line probe assay for detection of 37 HPV genotypes from cervical samples. Several studies have evaluated the proficiency of Linear Array for HPV detection (170–173). The Roche AMPLICOR test is comparable to LA; however, it can detect only 13 high-risk HPV types. When compared to its precursor, the line blot assay using PGMY primers, LA was found to be more sensitive and less specific, but had an improved rate of detection of multiple-genotype combinations and newer genotypes (171). Similar results were seen in studies comparing LA with SPF-10 InnoLiPA assay, where both the assays were equivalent and reproducible, but the LA was better at detecting multiple HPV types

(174). LA is also found to be superior to the Digene Hybrid Capture2 HPV kit in detection of carcinogenic HPV types (170). The Linear Array is therefore a thoroughly evaluated assay which is highly sensitive for the detection and genotyping of HPV, in particular multiple-genotype HPV infections. The main disadvantage of this assay is its cost (approximately Rs.4165/sample) which limits its use in low-resource settings.

Many laboratories have participated in the WHO HPV LabNet proficiency panel testing using CHUV assay but with varying results. Out of the five laboratories which participated in 2009, only two were fully proficient and one was proficient at 88% (173). Similar results were seen in the 2010 proficiency panel study where only four out of six studies were fully proficient (175). The performance of LA was also similar where only eight out of seventeen laboratories were fully proficient (175). This indicates that the results of testing with CHUV assay and the LA have not been consistent across laboratories. Compared to commercial assays, the PGMY-CHUV assay also requires a strict quality control similar to that required by any in-house assay.

Our study found the CHUV assay to have moderate agreement with the Linear Array for HPV detection and genotyping. Overall, the assay had good sensitivity (91%) but low specificity (52%). The sensitivity of CHUV was lower (77%) for clinical samples when compared to WHO samples (92%). Specificity was however higher in the WHO samples.

The PGMY09/11 primers are widely used and amplify a 450bp conserved region in L1 region of HPV genome. Other primer systems such as GP5+/6+ amplify a shorter fragment (150bp) of the HPV genome; however, the PGMY09/11 primers are equally sensitive for type-specific amplification and are better for identification of multiple HPV types in a single sample (130). Further, amplification using the PGMY primers can be detected using gel electrophoresis which is simple and less expensive. Similar line blot hybridisation assays using GP5+/6+ primers, employ enzyme immunoassays for detecting amplified products which is slightly more expensive (176). Independant of the CHUV genotyping results, HPV positivity with the PGMY primers in our study was 42% (21/50 clinical samples). However, more HPV positives were detected on further genotyping using the CHUV - RBH membrane. This implies that just the HPV DNA PCR in the PGMY-CHUV assay is not sufficient to stratify samples which require genotyping. This finding has been reported earlier. However, the CHUV PCR helps determine HPV positivity whereas LA lacks this step in testing and only direct reverse hybridisation gives a genotyping result.

Comparison of the genotyping results showed the rate of HPV positivity was 58% for CHUV which was much higher than 42% positivity by the Linear Array. Discordance was high in clinical samples, only 17 samples giving a positive result with both the assays. CHUV appeared to be more sensitive than LA, as it exclusively detected 10 clinical samples as HPV positive. Most of these samples had single HPV types and were reported negative by LA. There was no consistent pattern of discordance

between the samples. This may be explained partly by the lower specificity of LA which has been demonstrated in many studies (171,172). Szarewski et al. compared several adjuncts to cytology in patients with HSIL including LA which was found to have a specificity of 32.8%.

Among the 42 positive WHO samples, CHUV did not detect HPV in 6 samples; 3 of these samples had concentration less than 50 IU. This indicates that a false-negative result by CHUV may be due to lower concentrations of HPV in the samples.

Other factors that may have influenced the discordance between the two assays in the clinical samples are:

- Sampling errors: The cervical sample collected is only representative of the cervical epithelium and may contain only limited numbers of HPV-DNA copies, thus producing inconsistencies in an assay with good sensitivity. A study conducted by Quint et al., on HPV genotyping in cervical scrapes and biopsies demonstrated comparable but variable genotyping results (177).

- The sample collected is also influenced by the phase of menstrual cycle which can affect the outcome of results in terms of presence or absence of HPV DNA, the accuracy of HPV detection and the detection of multiple genotypes present at different concentrations (178,179).

- A recent study by Klug et al., compared various genotyping assays on the same set of cervical samples and clearly showed that the results are not fully congruent (180). This affects the comparison of newer assays with older licensed genotyping assays with regard to type-specific detection. This may have influenced the comparison of the results of CHUV and LA for the clinical samples.

The higher sensitivity of the CHUV assay in clinical samples may also partly be due to the method of post-amplification genotype detection by chemiluminescence, which is a more sensitive method of detection, when compared to colorimetric detection employed in Linear Array, and therefore a higher rate of positivity.

HPV genotypes prevalent in India include HPV-16, -18, -45, -33, -35, -58, -59 and -31 (60). The results of CHUV and LA showed a similar pattern of genotypes in the clinical samples. The range of genotypes identified by both the assays was comparable and included high-risk and low-risk types. LA exclusively identified HPV types -61, -62, -81 and CP6108. The CHUV reverse hybridisation panel does not contain probes for types -61, -62, -81 and CP6108 and hence could not detect these genotypes. CHUV genotyping of the WHO panel which consists of mixed combinations of HPV types was comparable to LA for most samples.

We included HIV-infected women in our study as they are reported to have infection with multiple HPV genotypes (8). One of the objectives of our study was to determine if CHUV was able to identify multiple-genotype combinations in samples. We found that although CHUV was slightly more sensitive than LA at overall HPV detection, it failed to detect multiple infections in as many as 6 clinical samples and 3 WHO samples that were identified by LA. No specific HPV types were consistently missed by CHUV in these samples. However, among the types missed in multiple infections were HPV-62 and -81 which are not included in the array of genotypes incorporated in

the CHUV probe array. The absence of certain HPV types in the CHUV panel compared to LA partly explains the lower detection rates of multiple infection.

Both LA and CHUV were able to identify high-risk types HPV-16 and -18. LA was more efficient in identifying type-16 (11 clinical samples), type -51 and -59 (5 clinical samples each). CHUV identified HPV-16 in 9 samples but was able to detect more number of HPV-31 (5 samples).

The ability of LA to detect HPV-52 especially in samples with mixed infections is low, however among the WHO samples, LA satisfactorily identified type-52 in single and multiple-genotype combinations. CHUV is reported to identify type-52 unambiguously unlike LA (169). This is important as HPV -52 is among the common genotypes prevalent in many regions in Asia, including India (60,181). In our study, CHUV identified type-52 in all single and multiple combinations in the WHO samples, except one sample with types -11,-18,-31 and -52 where it detected only type-11. HPV -52 was not detected by CHUV or LA in any of the clinical samples.

Two of our study samples, reported negative by LA, were PCR positive by the CHUV assay, although no genotype was detected on RBH. This may be due to presence of a genotype that is not a part of the CHUV genotype array. The CHUV assay, therefore, has an advantage over LA as the PCR amplicon is available for subsequent nucleotide sequencing to determine the genotype in these indeterminate samples. Sequencing can

also help in interpretation of samples positive only by the CHUV assay, in patients with normal cytology and clinical examination.

Repeat testing of all the exclusive positives in the CHUV assay must be considered.

Cost evaluation: Taking into account the extraction of DNA, PCR and genotyping, the cost of CHUV assay is estimated to be around Rs.1030 per sample. The main advantage of the CHUV assay is its reusable membrane and the projected reduction in cost of testing. This cost is much lower than the cost of Rs.4165 per sample for testing by the linear array.

Limitations of the study:

1. Larger sample size will help us assess the CHUV assay better.
2. Future evaluation studies could possibly include testing for reproducibility and nucleotide sequencing of discordant samples to determine the HPV genotype.

The results of our study show that the CHUV assay falls short of specificity and is poor in detecting multiple-genotype combinations. The ability of the assay to detect the full range of HPV types and better results observed with the WHO reference samples indicate it has potential for efficient genotyping.

Conclusion

India has a high burden of cervical cancer among women. Cervical cytology as a screening strategy and for diagnosis is effective but lacks sensitivity. As HPV has been implicated in the pathogenesis of cervical cancer, HPV DNA testing is now gaining importance in the screening and diagnosis of cervical cancer precursors. Many tests are available but are expensive. In a low-resource setting like ours, there is a need for a low-cost efficient testing method for HPV detection and genotyping.

We tested an in-house reverse hybridization assay in comparison to the commercially available Linear Array for HPV detection and genotyping. Samples were collected from 50 HIV-infected women as this group is known to have higher rates of HPV infection and multiple-genotype combinations. In addition, 43 WHO reference samples were also tested.

Overall, we found the CHUV assay to have an acceptable sensitivity but it lacked specificity for HPV detection. The rate of detection of multiple infection was also lower when compared to LA. The CHUV assay was, however, able to detect the entire range of genotypes in the WHO samples and this suggests that it can be an efficient tool for genotyping HPV. However, further studies with larger sample size are required to evaluate the proficiency of the assay and evaluate its suitability in a low-resource setting.

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Informed Consent

Christian Medical College, Vellore
Department of Microbiology

A pilot study to evaluate and establish the CHUV assay as a cost-effective tool for genotyping HPV from cervical smears of HIV-infected women.

Information sheet

Human Papilloma Virus (HPV) is a common virus that can infect humans. Infection occurs frequently by sexual transmission. Some HPV types can cause persistent infection of the cervix (lower part of the uterus). Persistent infection with HPV has been reported to increase the risk of cervical cancer. Screening tests for this infection in the cervix are available. Most of the tests to detect the presence of HPV infection are expensive and are therefore not used for routine screening of women in India. Newer cost-effective methods have been devised but have to be evaluated before use.
We are evaluating a new cost-effective test for the detection of this viral infection in cervical smears. Prospective study participants will be asked discretely about sexual activity over the last two years.
You are being requested to participate in a study to see if this new test can detect HPV infection as effectively as the standard established testing assay.

If you take part what will you have to do?

If you wish to participate in the study you will have to give consent for a pelvic examination and cervical smear which will be taken by your attending doctor. The smear will be tested for HPV infection and for cervical disease.

A blood sample will also be taken, the results of which will be informed to your doctor. This will be useful in the course of your subsequent treatment and follow-up.

Can you withdraw from this study after it starts?

Your participation in this study is entirely voluntary and you are also free to decide to withdraw permission to participate in this study. If you decide to withdraw from the study at any point of time, it will not affect your access to medical care in this hospital.

Will you have to pay for the cervical smear / assay & blood test?

The tests will be done free of cost.

Will the result of your test be informed to you?

The cervical smear results will be informed to your attending doctor only if cervical disease is suspected.

The results of the study will help us evaluate the new assay and if it performs well, it will be used in the future for detection of HPV infection in other women in the hospital.

However, the blood test will be reported and will be included in your medical record.

Will your personal details be kept confidential?

The results of this study will be kept confidential. Your samples will be coded and will be known only to the persons involved in the study.

Will your samples be used for other studies in the future?

We seek your permission to use your samples for evaluation of other cost-effective assays in the future studies. Confidentiality will be maintained as mentioned earlier.

If you have any further questions, please ask Dr.Pallavi Baliga (Mob No: +919629481442) or Dr.Priya Abraham (0416-2282312/2282070).

CONSENT TO TAKE PART IN A STUDY

Study Title: A study to evaluate and establish the CHUV assay as a cost-effective tool for genotyping HPV from cervical smears of HIV-infected women.

Study Number:
Participant's name:
Date of Birth / Age (in years):

I_____
_____, son/daughter of _____

(Please tick boxes)
Declare that I have read the information sheet provided to me regarding this study and have clarified any doubts that I had. [ ]
I also understand that my participation in this study is entirely voluntary and that I am free to withdraw permission to continue to participate at any time without affecting my usual treatment or my legal rights [ ]
I also understand that during the study, the cervical smears and the tests done on the specimen will be done free of cost.
I understand that the study staff and institutional ethics committee members will not need my permission to look at my health records even if I withdraw from the trial. I agree to this access [ ]
I understand that my identity will not be revealed in any information released to third parties or published [ ]
I understand that my cervical smear may be used in future studies for evaluation of other cost-effective assays [ ]
I voluntarily agree to take part in this study [ ]

Name:
Signature:
Date:

Name of witness:
Relation to participant:
Date:

Patient Proforma

Christian Medical College, Vellore
Department of Microbiology

A pilot study to establish and evaluate the CHUV assay as a cost-effective tool for genotyping human papillomavirus from cervical smears of HIV-infected women.

A. Patient Details:

1. Name of patient :
2. Age:
3. Hospital No:
4. Address:

5. Marital Status (married/unmarried/widow):

6. Parity:

7. Symptoms- a) None b) WDPV

   c) IMB/PCB d) Others

8. Age of onset of sexual activity :

9. No. of lifetime sexual partners :

10. Symptoms of STI (Yes/No/Dont Know) :
    a) Genital ulcer b) Dysuria
    c) Urethral discharge d) Others
    e) Past h/o STI –

B. Partner/Husband details (If available) -

1. HIV status –
2. No. of lifetime sexual partners:
3. Symptoms of STI (Yes/No/Dont Know):

C. HIV infection in Patient:

1. When was HIV infection diagnosed? (Date of diagnosis):
2. Place of Diagnosis of HIV infection:
3. WHO staging of Disease:

   Remarks (Staging) –

D. CD4+ count:

E. Pap smear report of patient:

1. Normal
2. Koilocytosis
3. Mild dysplasia
4. Moderate dysplasia
5. Severe dysplasia

E. HPV infection in patient:

1. Present/Absent:
2. HPV type:

F. Any other STI(s) diagnosed at present:

ANNEXURE
Procedures

1. Preparation of TE Buffer

- 10 mM Tris Cl (pH 8.0 and pH 7.0)
- 1mM EDTA (pH 8.0)
- Dissolve in 100mL distilled water.

2. Preparation of TAE buffer (50X)

- Tris base - 242 g
- Glacial acetic acid - 57.1 mL
- 0.5 M EDTA - 100 mL (pH 8.0)

3. Ethidium Bromide (10 mg/mL, Stock)

- Ethidium bromide - 1g
- Milli Q H_2O - 100 mL

4. Gel loading buffer

- 0.25% bromophenol blue
- 40% (w/v) sucrose in water
- Stored at 4^oC.

DISSERTATION ABSTRACT

TITLE OF THE ABSTRACT : A pilot study to establish and evaluate the CHUV assay as a cost effective tool for genotyping human papillomavirus in HIV-infected women

DEPARTMENT : Department of Clinical Microbiology

NAME OF THE CANDIDATE : Dr.Pallavi Ravindra Baliga

DEGREE AND SUBJECT : MD Microbiology

NAME OF THE GUIDE : Dr. Priya Abraham, Professor, Department of Clinical Virology

OBJECTIVES:

1. To establish a cost-effective, in-house reverse hybridization Assay (CHUV assay) as a tool for HPV detection and genotyping in HIV-infected women.
2. To compare the accuracy indices of the CHUV assay with a licensed commercial assay (Linear Array, Roche Diagnostics).

METHODS:

Study design: Prospective study of diagnostic test accuracy.
Study duration: March 2011-July 2012
Study samples: Cervical brush samples from 50 HIV-infected women and 43 WHO reference samples (HPV purified plasmid DNA). All samples were tested by both assays. PCR followed by genotyping was done for all samples.
Index test: CHUV reverse hybridization assay – genotype detection is by autoradiography in a dark room.
Gold standard: Linear Array (Roche Diagnostics) -genotype detection is by colorimetric detection.
The Accuracy indices – sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated with a 95% confidence interval for the CHUV assay in comparison to the Linear Array

RESULTS AND CONCLUSIONS:

The CHUV assay in comparison to the Linear Array showed a sensitivity of 91%, specificity of 52% and a moderate agreement for all samples that were compared. It was not efficient in detecting multiple-genotype combinations. However, most high-risk HPV types were identified and the entire range of genotypes in the WHO reference panel was detected.

The CHUV assay had an acceptable sensitivity but it lacked specificity for HPV detection. Despite the lower rates of detection of multiple infection, satisfactory results were obtained with the WHO reference samples and the ability of the assay to identify the entire range of genotypes suggests that it can be an efficient tool for genotyping.